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Isolation and Characterization of a Glycerol Auxotroph of *Rhodopseudomonas capsulata*: Effect of Lipid Synthesis on the Synthesis of Photosynthetic Pigments

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A glycerol auxotroph was isolated from *Rhodopseudomonas capsulata* for use as a system for studying membrane synthesis and function. When the mutant was deprived of glycerol, net phospholipid synthesis ceased immediately and a small amount of free fatty acids accumulated. A turnover of lipid occurred in both deprived and supplemented cultures. Deoxyribonucleic acid and protein synthesis continued for one doubling of cell mass and then slowed down in deprived cells. Net ribonucleic acid synthesis slowed down more dramatically. Oxidative phosphorylation activity of membrane preparations from aerobically and semianaerobically grown cells appeared unaffected by glycerol deprivation, indicating that simultaneous lipid synthesis is not a requirement for new oxidative phosphorylating activity. In the absence of net phospholipid synthesis, bacteriochlorophyll and carotenoid syntheses were reduced to 30% of the activity of supplemented cultures. δ-Aminolevulinic acid synthase, the first enzyme on the bacteriochlorophyll pathway that is subject to regulatory control, increased in activity in deprived cultures. Lascelles and Szilagyi (1965) showed an association between phospholipid synthesis and pigment production. They found an increased lipid content associated with pigmented cells. The present results indicate that not only is there an association between lipid and pigment synthesis, but also there is actually a dependence of bacteriochlorophyll synthesis on phospholipid synthesis.

Rhodopseudomonas capsulata is a member of the family *Athiorhodaceae* and like other members of this family, has an extensive system of intracellular photosynthetic membranes (4). The intracellular membranes appear in the form of vesicles called chromatophores, which contain the bacteriochlorophyll (BChl) and light-harvesting pigments of the cell (11). When *R. capsulata* is grown aerobically and heterotrophically, intracellular membranes are not present, but chromatophores are formed when the oxygen tension is lowered (6). Thus, *R. capsulata* provides an interesting system for studying membrane biogenesis. We have isolated a glycerol-requiring mutant of *R. capsulata* that should be helpful in elucidating the role of the lipid in the formation and functioning of chromatophores. This paper describes the isolation and characterization of this mutant and the effects of the cessation of lipid synthesis on the formation of carotenoids and BChl.

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MATERIALS AND METHODS

Media. Cells were grown at 30°C in Lascelles medium S (17) or in RCV-B medium of Ormerod et al. (33) supplemented with a mixture of pure amino acids approximating 0.5 mg of casein hydrolysate per ml. Media for the growth of glycerol-requiring mutants contained 20 μg of glycerol per ml. Cultures were incubated aerobically in a rotary shaking bath in flasks of a capacity 10 times that of the culture volume. Cultures were incubated semi-anaerobically in 100-ml tubes fitted with bubblers. A mixture of 2 or 3% oxygen obtained by mixing N_2 and air through a Matheson gas proportionater model 665 was bubbled through the culture at a flow rate of approximately 80 cm^3/min. All gases used were of prepurified grade. Photosynthetic cultures were grown in 100-ml tubes fitted with bubblers through which a mixture of N_2 and CO_2 was added. Illumination (800 ft-c [ca. 8,608 lx]) was provided by a bank of 60-W tungsten lamps.

Strains and mutagenesis. *R. capsulata* was obtained from G. Sojka. Aerobic cells growing loga-

rithmically on Lascelles medium were treated with 0.8% (vol/vol) ethyl methane sulfonate (Eastman) for 30 min, centrifuged, washed, resuspended on growth medium supplemented with 20 μg of glycerol per ml, and incubated overnight. On the next morning, the cultures were diluted and incubated until several doublings of cell number occurred. Glycerol auxotrophs were isolated by replica plating. Cells were plated on Lascelles medium with 20 μg of glycerol per ml, incubated for 48 h, and then replicated onto plates with and without glycerol. Colonies that appeared on glycerol-supplemented plates but not on unsupplemented ones were picked and purified. The first glycerol mutant isolated was designated C2.

Growth experiments. Growth was measured as optical density at 680 nm in the Coleman Junior spectrophotometer. Cultures were switched from glycerol to no-glycerol conditions by filtering and washing cells on HAWP 0.45-μm membrane filters (Millipore Corp.) and resuspending the cells in prewarmed medium.

Isotope experiments. ^{14}C- and ^{3}H-labeled acetate and uridine were obtained from New England Nuclear Corp. ^{32}P-labeled phosphate was obtained from New England Nuclear Corp. and was purified by filtration for lipid uptake studies. For oxidative phosphorylation assays, ^{32}P was purified by ashing followed by heating in 1 N HCl for 15 min. Radioactivity was determined in a Beckman LS200B scintillation counter in vials containing 10 ml of Aquasol (New England Nuclear Corp.). Cerulenin was a gift from S. Omura.

Analytical methods. Protein was measured with the Folin phenol reagent, using bovine serum albumin as a standard (24). Ribonucleic acid (RNA) was measured by the orcinol method, using adenosine as the standard (27). Deoxyribonucleic acid (DNA) was determined by a modification of the diphenylamine method (8). Samples (20 ml) were removed from a growing culture and precipitated with cold trichloroacetic acid. Samples were centrifuged and the pellets were resuspended in 2.5 ml of 5% trichloroacetic acid, heated at 90°C for 10 min, cooled, and centrifuged. RNA and DNA determinations were made on the hot acid-soluble fraction while the protein content of the pellet was measured.

BChl was measured by extraction with acetone-methanol by the procedure of Cohen-Bazire et al. (4). The absorbancy of the extract was measured at 775 nm, using an extinction coefficient of 75 mM^{-1}/cm to calculate BChl per milliliter of culture (3). BChl determinations were also made by the method of Sojka et al. (37), which involved measuring the absorption of a suspension of whole cells in 25% bovine serum albumin at 860 and 660 nm. Carotenoids were measured by the procedure of Cohen-Bazire et al. (4), which involved measuring the absorption of an acetone-methanol extract of cells at 456 and 510 nm and correcting for absorption by BChl. All spectrophotometric measurements were made with the Zeiss spectrophotometer PMQ II.

Incorporation of [^{14}C]acetate or ^{32}P-labeled phosphate into lipid. Cultures were grown overnight in 10 mM acetate and then filtered, washed, and resuspended in 10 mM acetate and 1 μCi of [^{14}C]acetate per ml. In some experiments, cultures were prelabeled overnight with the radioactive compound. In this case, cells were resuspended in medium containing the labeled compound at the same specific activity as the prelabeled cells. The cultures were incubated with or without glycerol, and 1- or 2-ml samples were taken for extraction by the procedure of Bligh and Dyer (2). In some cases 1×10^8 to 2×10^8 carrier cells were added before extraction.

The lipid extracts dissolved in chloroform-methanol (2:1) were chromatographed on Whatman silica gel-loaded paper and on Brinkman thin-layer silica gel plates. Silica gel plates were run in petroleum ether-diethyl ether-acetic acid (70:30:2) (36), and the SG-81 paper was run in chloroform-methanol-diisobutyl ketone-acetic acid-water (45:15:30:20:4) (39). Standard mixtures containing mono-, di-, and triglycerides and palmitic acid (Applied Science Laboratories; TLC-8) or β-hydroxy, β-keto, and α-β-unsaturated fatty acids (gift from M. Pullman) were run along with radioactive samples on silica gel plates.

Autoradiography was carried out by the application of Kodak No-Screen X-ray film for 3 days. The film was developed and radioactive spots were scraped off the thin-layer plates and counted in Aquasol (New England Nuclear Corp.). Radioactive spots were cut out of the paper and counted in 5 ml of toluene cocktail consisting of 4 g of PPO (2,5-diphenyloxazole) and 0.1 g of POPOP [1,4-bis-(5-phenyloxazolyl)benzene] per liter of toluene. The uptake of [^{32}P]phosphate into lipid was carried out in the same manner.

Incorporation of [^{3}H]uridine into RNA. C2 was grown overnight in medium containing 20 μg of uridine per ml, filtered, washed, resuspended in medium with 1 μCi of [^{3}H]uridine per ml and 20 μg of unlabeled uridine per ml, and incubated with and without glycerol. Samples (0.2 ml) were taken, precipitated with trichloroacetic acid, and washed, and the filters were counted in 5 ml of Aquasol.

Assay of oxidative phosphorylation. Cells grown aerobically or semi-anaerobically were harvested by centrifugation and resuspended in 0.05 M phosphate buffer (pH 7.0) or in 0.05 M glycylglycine buffer (pH 7.2)-2.5 mM $MgCl_2$. Cultures were lysed by passage through an Aminco French pressure cell at 10,000 lb/in^2 (703 kg/cm^2). Lysates were centrifuged at low speed to remove debris, and membranes were pelleted at 105,651 $\times g$. The pellet, the crude membrane preparation, was resuspended in 0.05 M phosphate (pH 7.0), 1 mM $MgSO_4$, or 0.1 M glycylglycine buffer (pH 7.2)-2.5 mM $MgCl_2$ and used for oxidative phosphorylation assays. Crude membrane preparations were kept frozen until use.

Oxidative phosphorylation was assayed by the procedure of Baccarini-Melandri and Melandri (1), which involved the use of a hexokinase plus glucose trap to measure the conversion of [^{32}P]adenosine 5'-triphosphate (ATP) to [^{32}P]glucose-6-phosphate. Samples (1 ml) of crude membrane preparation were added to Corex tubes containing the following reaction mixture: 0.125 ml of 0.8 M glycylglycine-NaOH buffer, pH 8.0, 0.03 ml of 0.5 M $MgCl_2$, 0.05 ml of 0.5

M D-glucose, 0.06 ml of 0.05 M adenosine 5'-diphosphate, 0.011 ml of 0.2 M sodium succinate, 0.02 ml of sulfate-free hexokinase (Sigma Chemical Co., St. Louis, Mo.) (250 U/ml), and 0.1 ml of 0.15 M Na_2HPO_4 containing ^{32}P (1 μCi of Na_2HPO_4 per 15 μmol). Incubation was carried out at 30°C for the indicated time, and the reaction was stopped by the addition of 0.1 ml of 35% perchloric acid. The reaction mixture was extracted by the procedure of Pullman (34). A 1-ml amount of the reaction mixture was added to 2 ml of 2.5 N perchloric acid and 5 ml of isobutanol-benzene (1:1). A 1-ml amount of 5% ammonium molybdate was immediately added to the tube. The tube was vortexed twice for 10 s, the upper phase was removed, and the lower phase was reextracted with 5 ml of isobutanol saturated with water. The extraction was repeated. A 1-ml amount of the aqueous phase was counted in 10 ml of Aquasol. A zero-time control was run in this assay, and total counts were determined in a control tube containing all components of the reaction mixture except the crude membrane preparation. A 1-ml amount of the supernatant from the assay was mixed with 1 ml of isobutanol-saturated water, and total counts were determined by counting 1 ml of the mixture in 10 ml of Aquasol. The micromoles of ATP formed = corrected counts per minute × volume × micromoles of inorganic orthophosphate in the tube per the total counts per minute.

Incorporation of [^{14}C]glycine and [^{14}C]ALA into BChl. When [^{14}C]δ-aminolevulinic acid (ALA) was used to label BChl, cultures were grown overnight semi-anaerobically in RCV-B medium containing 1 mM ALA. Cells were filtered, washed, resuspended in media containing 1 mM ALA and 1 μCi of [^{14}C]ALA with and without glycerol, and incubated semi-anaerobically. In certain cases, cells were labeled overnight and then resuspended in medium of the same specific activity. Samples (1 ml) were taken for BChl extraction with acetone-methanol by the method of Cohen-Bazire et al. (4), and the extracts were evaporated to dryness, resuspended in a small amount of acetone-methanol, and applied to a Brinkmann silica gel plate. Chromatography was carried out in chloroform-methanol-water (150:50:4). Autoradiography was carried out and spots corresponding to BChl were scraped and counted. The uptake of [^{14}C]glycine into BChl was carried out in the same manner, except that no cold glycine was added since the media contained 6.2 mM glycine. A 1-μCi amount of [^{14}C]glycine per ml was used to label BChl.

Assay of ALA synthase. Cell extracts were prepared as described for the assay of oxidative phosphorylation, except that the high-speed supernatant fluid was saved instead of the pellet. ALA synthase was assayed by the procedure of Kikuchi et al. (15). ALA was estimated colorimetrically after condensation with acetylacetone by the method of Gibson et al. (7). The assay tube contained 0.1 ml of 1 M phosphate buffer, pH 7.4, 50 μmol of succinate, 50 μmol of glycine, 1 μmol of coenzyme A, 0.25 μmol of pyridoxal PO_4, 5 μmol of ATP, 5 μmol of $MgCl_2$, 0.025 μmol of $MnSO_4$, 1.5 μmol of glutathione, and 1 ml of soluble extract. Incubation was carried out at 34°C for 1 to 2 h. The reaction was stopped by the addition of 0.2 ml of 50% trichloroacetic acid, and the precipitated protein was removed by centrifugation. A 1-ml amount of the supernatant, 1 ml of 1 M acetate buffer, pH 4.6, 0.1 ml of 2.5 N NaOH, and 0.05 ml of acetylacetone were heated at 100°C for 10 min and cooled, and 2 ml of modified Ehrlich reagent was added. Modified Ehrlich reagent was prepared by dissolving 1 g of *p*-dimethylaminobenzaldehyde in glacial acetic acid and perchloric acid (26). Internal standards were included to determine micromoles of ALA formed. After 15 min, the optical density at 555 nm was read.

RESULTS

General properties of the mutant C2. A glycerol auxotroph designated C2 was isolated from *R. capsulata* as described above. C2 grew normally, with a generation time of approximately 180 min if supplemented with glycerol at a concentration of 20 μg/ml. When a logarithmically growing culture of C2 was deprived of glycerol, growth continued for approximately one generation time at a progressively slower rate. DNA and protein synthesis continued for about 3 h after deprival and then leveled off, but net RNA synthesis slowed down more dramatically (Fig. 1). The addition of [^{3}H]uridine at the time of deprival resulted in the accumulation of label by deprived and supplemented cells (unpublished data), indicating that although net RNA synthesis ceased upon depri-

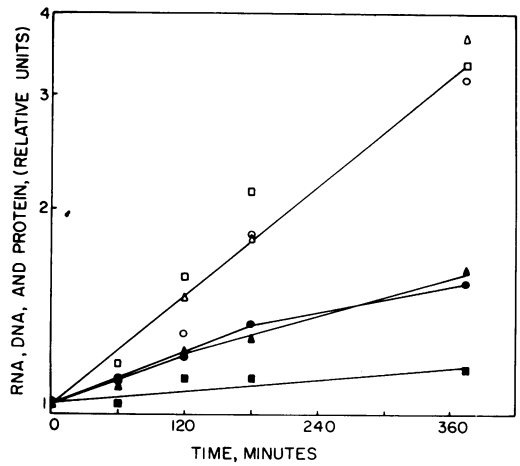

FIG. 1. *Effects of glycerol deprival on RNA, DNA, and protein synthesis. An exponentially growing aerobic culture of C2 in RCV-B medium was filtered, washed, and resuspended in 100 ml of RCV-B medium with (open symbols) and without (closed symbols) glycerol. RNA* (□, ■), *DNA* (△, ▲), *and protein* (○, ●) *were determined as described in the text. The resulting values were normalized to facilitate comparison.*

vation, turnover and synthesis of RNA were occurring.

Effect of glycerol deprivation on phospholipid synthesis. The utility of the glycerol auxotroph C2 depended on the demonstration that the removal of glycerol results in the cessation of phospholipid synthesis. The uptake of [^{14}C]acetate into phospholipid was measured in deprived and supplemented cells. Net phospholipid synthesis was measured by prelabeling cells in medium containing [^{14}C]acetate and then switching to deprived and supplemented conditions in media of the same specific activity. Figure 2A shows that net phospholipid synthesis ceased immediately after glycerol deprival. The same results were obtained when cells were prelabeled with [^{32}P]phosphate (not shown). Figure 3A shows that label was incorporated by both deprived and supplemented cells, although supplemented cultures incorporated three times as many counts into phospholipid as did deprived cells. Mindich (28) showed that the turnover of the phosphate moiety in *Bacillus subtilis* lipids occurred in the presence or absence of glycerol. Figure 4 shows that the turnover of fatty acids in phospholipid occurs in deprived and supplemented cells of *R. capsulata*, and this could account for the incorporation of label by deprived cells.

Effect of glycerol deprival on free fatty acid synthesis. It was of interest to determine the effect of glycerol deprivation on fatty acid synthesis in C2 since in the absence of phospholipid synthesis there would be no acceptor for the transacylation step. Figure 3B shows that a small number of counts are incorporated into free fatty acids by deprived cells when label is added at the time of glycerol removal. A net increase in free fatty acids is occurring, as evidenced by the accumulation of counts in prelabeled cells (Fig. 2B).

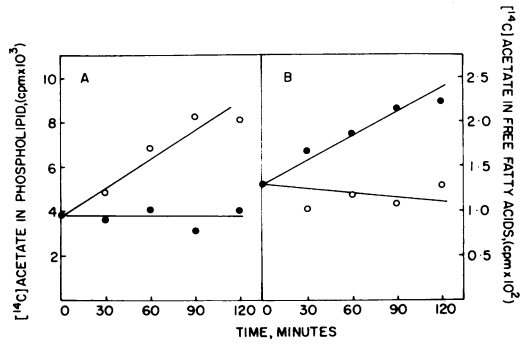

FIG. 2. *[^{14}C]acetate uptake into phospholipid and free fatty acids. A culture of C2 was grown aerobically overnight in 100 ml of RCV-B medium containing 100 μCi of [^{14}C]acetate, filtered, washed, resuspended in RCV-B and [^{14}C]acetate at the same specific activity, and incubated aerobically with* (○) *or without* (●) *glycerol. The lipids from 2-ml samples and 2 ml of carrier cells (1.2 × 10⁸ cells/ml) were extracted and chromatographed on silica gel plates as described in the text. (A) [^{14}C]acetate into phospholipid; (B) [^{14}C]acetate into free fatty acids.*

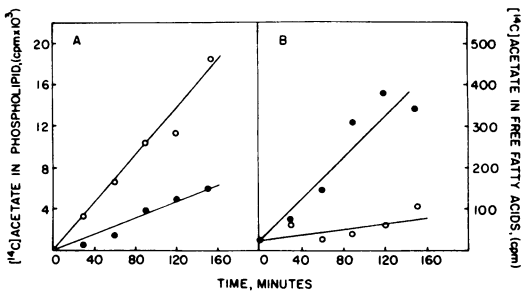

FIG. 3. *[^{14}C]acetate uptake into lipid by deprived and supplemented cultures. A culture of C2 was grown aerobically overnight in RCV-B medium containing 10 mM acetate, filtered, washed, and resuspended in 30 ml of medium with 10 mM acetate and 30 μCi of [^{14}C]acetate with* (○) *or without* (●) *glycerol. Samples (2 ml) were extracted by the method of Bligh and Dyer (2), and the extracts were chromatographed on silica gel plates, as described in the text. (A) [^{14}C]acetate into phospholipid; (B) [^{14}C]acetate into free fatty acids.*

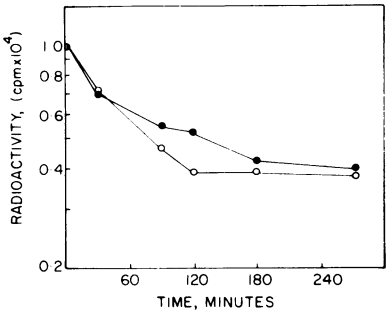

FIG. 4. *Turnover of [^{14}C]acetate in lipids of C2. Cells were grown overnight in RCV-B medium with 1 μCi of [^{14}C]acetate per ml, washed, and resuspended in RCV-B medium with 20 μg of glycerol per ml* (○) *or without glycerol* (●). *Samples were removed at indicated times and the lipids were extracted (2).*

Effect of deprivation on oxidative phosphorylation. To determine whether the cessation of growth after glycerol deprival was the result of a defective oxidative phosphorylation system, we looked at oxidative phosphorylation activity in the absence of net phospholipid synthesis. Figure 5 shows that membrane preparations isolated from cells grown semi-anaerobically with and without glycerol continued to incorporate radioactivity into ATP. The potential for oxidative phosphorylation remained at a simi-

lar level of specific activity in deprived and supplemented cells.

Effect of deprivation on ALA synthase activity. ALA synthase is the first enzyme on the BChl pathway and is subject to regulatory control. Gorchein et al. (10) reported that pigment induction in *R. spheroides* is accompanied by a 10-fold increase in ALA synthase when aerobic cells are switched to photosynthetic growth conditions. Lascelles (18) has shown that adaptation from aerobic to semianaerobic conditions resulted in a fourfold increase in the level of ALA synthase. BChl synthesis has been associated with phospholipid synthesis, as pigmented organisms have been found to contain a higher concentration of phospholipid (22). Soluble extracts were prepared from deprived and supplemented cells to determine the effect of cessation of phospholipid synthesis on ALA synthase activity. ALA synthase activity increased in both semianaerobic and photosynthetic deprived and supplemented cells (Fig. 6A). ALA synthase specific activity remained constant (Fig. 6B),

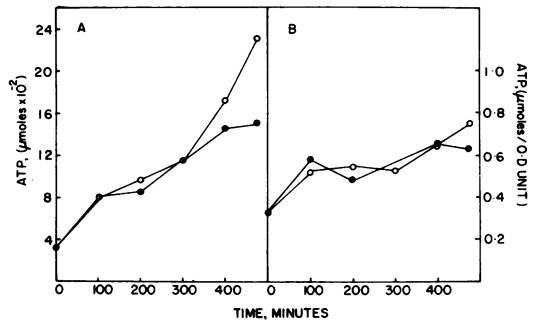

FIG. 5. *Oxidative phosphorylation by membrane preparations from semianaerobic cells. Oxidative phosphorylation was assayed, as described in the text, in semi-anaerobically grown cultures with* (○) *and without* (●) *glycerol. OD, Optical density.*

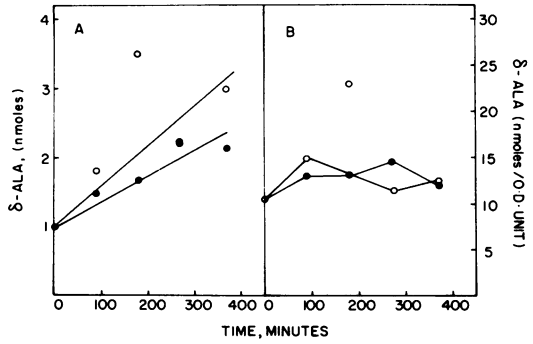

FIG. 6. *ALA synthase in semianaerobic cultures. ALA synthase was assayed in soluble extracts prepared from semi-anaerobically grown cultures with* (○) *or without* (●) *glycerol. OD, Optical density.*

indicating that ALA synthase was being induced in the absence of lipid synthesis. Figure 6 shows the results for semianaerobic cultures; those for photosynthetic cultures were similar.

Effect of glycerol deprivation on BChl synthesis. Spectral measurement of BChl in a culture deprived of glycerol indicated that BChl synthesis was affected by glycerol deprivation. Although the BChl content of deprived cells doubled during a 330-min period, the BChl content of supplemented cells increased by 10-fold. After normalization to growth rate, the rate of BChl synthesis of supplemented cells was 2.8 times the rate of BChl synthesis of deprived cells. The readdition of glycerol to cells deprived for 135 min resulted in the resumption of BChl synthesis at the same rate of synthesis as in the supplemented culture (Fig. 7). After a 215-min incubation in the presence of glycerol, these cells had a BChl content of 3.3 μg/mg of protein. Supplemented cells contained 5.5 μg of BChl per mg of protein, whereas the pigment content of deprived cells was 2.05 μg/mg of protein. BChl was also measured spectrally in cell-free fluid to determine whether pigment was being excreted by deprived cells. The low level of BChl in deprived cultures could not be explained by pigment excretion since none was detected in the culture medium under conditions in which 5% of the cellular content could have been measured.

BChl was labeled with [^{14}C]glycine or [^{14}C]ALA to determine whether turnover was important in the apparent decrease in the rate of BChl synthesis. Cultures supplemented with glycerol incorporated twice as many [^{14}C]glycine counts into BChl as did deprived cultures (Fig. 8). As a control, after 165 min of incubation in medium containing [^{14}C]glycine,

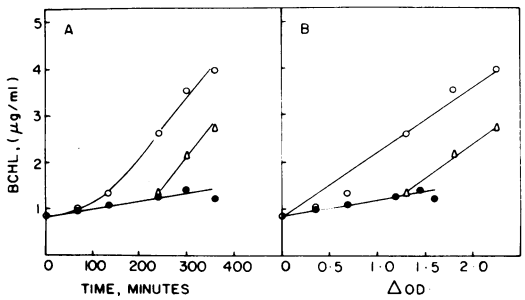

FIG. 7. *Effect of readdition of glycerol on BChl synthesis. A semi-anaerobically growing culture was filtered, washed, and resuspended with* (○) *or without* (●) *glycerol. Samples (5 ml) were removed for BChl determinations by the method of Cohen-Bazire et al. (4). After 135 min, 20 μg of glycerol per ml was added back to half of the deprived culture* (△). *OD, Optical density.*

aerobic cells incorporated 10% of the total counts incorporated by a semianaerobic culture into BChl. When normalized against optical density, the rate of incorporation of counts into BChl by supplemented cells was 2.2 times the rate of BChl synthesis by deprived cultures (Fig. 8). Cells, prelabeled in [^{14}C]glycine and switched to deprived and supplemented conditions in media containing the same specific activity, also showed a larger number of counts incorporated by the supplemented cells (data not shown). The addition of [^{14}C]ALA to a semianaerobically growing culture resulted in a slower rate of incorporation into BChl by the deprived cells than by the supplemented cells. When normalized to optical density, supplemented cells incorporated three times as many counts into BChl as did deprived cells.

Lascelles (18) reported an obligatory coupling between BChl and protein synthesis. Pigment synthesis is inhibited by amino acid deprivation and inhibitors of protein or nucleic acid synthesis. It was of interest to determine whether deprived cells could utilize proteins made during glycerol starvation for BChl synthesis. A culture of C2 was deprived of glycerol for 150 min and then chloramphenicol and glycerol were added back simultaneously. After 375 min of incubation, the BChl content in the supplemented culture increased 4-fold, whereas there was a 1.4-fold increase in the deprived culture. In the presence of chloramphenicol, the readdition of glycerol to a deprived culture did not result in the resumption of BChl synthesis at the supplemented rate.

Effect of glycerol deprivation on carotenoid synthesis. Carotenoid synthesis accompanies BChl synthesis and is usually roughly correlated to the concentration of BChl (32). The effect of cessation of phospholipid synthesis on carotenoid synthesis was investigated in deprived and supplemented cells. When red carotenoid synthesis was measured spectrophotometrically, it was found that there was a 4-fold increase in the carotenoid content of supplemented cells in a 330-min period, whereas there was a doubling of content in the deprived cells during the same interval (Fig. 9A). The rate of red carotenoid synthesis in supplemented cells was about three times the rate in deprived cells after normalization for cell mass (Fig. 9B).

Addition of cerulenin to wild-type culture. The addition of the antibiotic cerulenin inhibits the growth of *Escherichia coli* by blocking lipid synthesis (9). It was of interest to determine whether cerulenin also affects lipid synthesis in *R. capsulata*. If cerulenin blocked lipid synthesis in the wild type, would it affect BChl and protein synthesis in the same manner as glycerol starvation affects C2?

The addition of 100 μg of the antibiotic cerulenin per ml to an exponentially growing culture of wild-type *R. capsulata* resulted in the cessation of net lipid synthesis (Fig. 10B). Protein synthesis as measured by [^{14}C]glycine incorporation into hot trichloroacetic acid-precipitable material continued for at least 40 min after the addition of cerulenin. BChl synthesis, measured by incorporation of [^{14}C]glycine counts into the Bchl spot, was inhibited by the addition of cerulenin. The rate of BChl synthesis in the presence of cerulenin is 40% of the control rate when corrected for differing rates of protein synthesis (Fig. 10A).

DISCUSSION

The results of glycerol starvation on growth appear quite similar for the glycerol auxotrophs

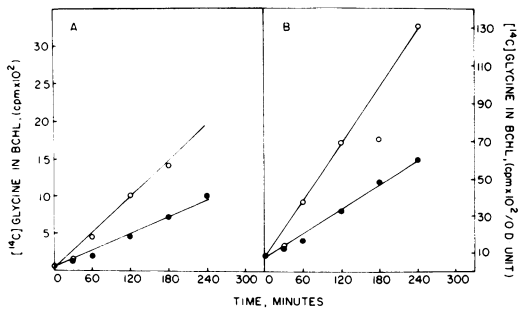

FIG. 8. *Effect of glycerol deprivation on [^{14}C]glycine uptake into BChl. A culture of C2 was grown semi-anaerobically in RCV-B medium, filtered, washed, and resuspended in RCV-B medium containing 1 μCi of [^{14}C]glycine per ml with* (○) *or without* (●) *glycerol. Samples (1 ml) were extracted for BChl determinations as described in the text. OD, Optical density.*

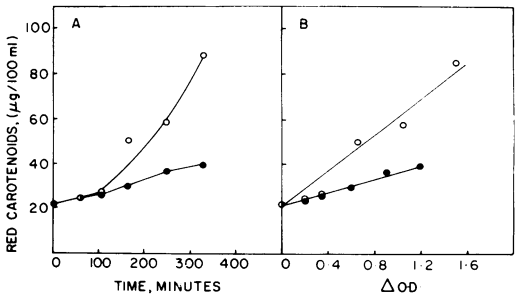

FIG. 9. *Effect of glycerol deprivation on carotenoid synthesis. A semi-anaerobically growing culture was filtered, washed, and resuspended in RCV-B medium with* (○) *or without* (●) *glycerol. Samples (5 ml) were taken for carotenoid determinations by the method of Cohen-Bazire et al. (4). OD, Optical density.*

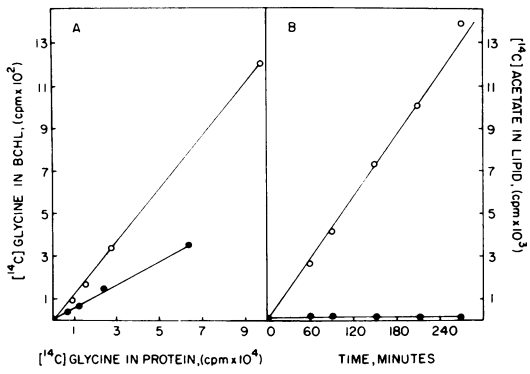

FIG. 10. *Effect of cerulenin on wild-type R. capsulata. A total of 100 μg of cerulenin per ml was added to an exponentially growing culture of R. capsulata. (A) [14C]glycine uptake into BChl was measured with (●) and without (○) cerulenin. [14C]glycine incorporation into hot trichloroacetic acid-precipitable material was determined as a measure of protein synthesis. (B) [14C]acetate incorporation in the presence (●) and absence (○) of cerulenin was followed as described in the text.*

of *B. subtilis*, *Staphylococcus aureus*, *E. coli*, and *R. capsulata* (31). The glycerol auxotrophs of *B. subtilis* go through one doubling of cell mass and then stop growing (28). *S. aureus* and *E. coli* also stop growing after one division (12, 29). In the absence of glycerol, C2 continues to grow for one generation time and then the growth slows down. This inhibition of growth can be reversed by the addition of glycerol, indicating that the cells remain viable in the absence of net phospholipid synthesis.

The effects of glycerol deprival of C2 on DNA, RNA, and protein synthesis are similar to the effects found by Mindich (28) with the glycerol auxotrophs of *B. subtilis*. The effect of deprivation on growth as measured by optical density followed the effect of glycerol starvation on protein synthesis. After glycerol removal, DNA and protein synthesis continued for one generation time in both *B. subtilis* and C2 and then slowed down. Net RNA synthesis shut off early in both mutants. However, Mindich (28) showed that messenger RNA synthesis continued in *B. subtilis*, as evidenced by continued protein synthesis and the ability to induce several enzymes. [3H]uridine uptake by deprived cultures of C2 in the absence of net RNA synthesis indicated that turnover and synthesis of RNA were occurring in the absence of phospholipid synthesis.

The removal of glycerol from cultures of C2 caused the immediate cessation of net phospholipid synthesis. The phosphate moiety of *B. subtilis* lipids turns over in the presence or absence of glycerol, whereas the fatty acids in the phospholipids of the gram-positive organisms *S. aureus* and *B. subtilis* were quite stable (28). In C2, the turnover of fatty acids in phospholipid also occurred in the deprived and supplemented state.

In the absence of glycerol, *B. subtilis* glycerol auxotrophs continued to synthesize fatty acids, which accumulate in the free form (30). In *S. aureus* there also appeared to be no regulation of fatty acid synthesis by the feedback of free fatty acids (29). Although the apparent rate of synthesis in these two gram-positive organisms was reduced, fatty acid synthesis was actually occurring at a normal rate. The apparent diminished rate was due to the high rate of turnover of the free fatty acids.

When C2 was deprived of glycerol, only a small amount of free fatty acids accumulated. This was similar to the situation that occurred with *E. coli* and was in marked contrast to the gram-positive bacteria *B. subtilis* and *S. aureus*, which accumulated large quantities of free fatty acids in the absence of phospholipid synthesis. Fatty acid synthesis of the two gram-negative organisms appeared to be regulated by feedback inhibition. It is possible that the acyl carrier protein derivatives of fatty acids are more stable in gram negatives than in gram positives, and fatty acid synthesis might be limited by a lack of free acyl carrier protein (30, 31). *E. coli* has two modes of control; when grown on glucose there is little free fatty acid formed, but when grown on succinate an activity is induced after glycerol deprival that leads to free fatty acid formation (5).

Klemme et al. (16) showed that phospholipase treatment of chromatophores of *R. rubrum* resulted in the almost complete loss of photophosphorylation activity and the ATP-inorganic orthophosphate-exchange reaction, whereas adenosine triphosphatase activity and inorganic pyrophosphatase activity were inhibited by 70%. Therefore, we examined the ability of deprived and supplemented cultures to carry out oxidative phosphorylation. In the absence of net phospholipid synthesis, oxidative phosphorylation activity continued to increase, indicating that simultaneous lipid synthesis was not a requirement for new oxidative phosphorylating activity. Membranes of deprived cells appeared to have a functional oxidative phosphorylating system. It appears that the defect that causes the cessation of growth of glycerol auxotrophs does not involve the structure of the oxidative phosphorylation system. Phospholipase treatment and acetone extraction of lipids are harsh methods of removing lipid. More extensive removal of lipid from C2 might well affect oxidative phosphorylation.

When photosynthetic bacteria are switched from aerobic to semianaerobic growth conditions, BChl synthesis is induced and chromatophores appear in the cell. Along with the induction of BChl, carotenoids also increase (4, 25). Lascelles (18) has shown that adaptation from aerobic to semianaerobic conditions results in a fourfold increase in the level of ALA synthase, the first enzyme of the BChl pathway. It has generally been accepted that BChl formation is associated with membrane formation.

A number of investigators have reported that an increase in the phospholipid content accompanies pigmentation. Steiner et al. (38) found that in *R. capsulata* a doubling in BChl is accompanied by a 20% increase in total phospholipid. They observed that the composition of the chromatophore membrane differed from the cytoplasmic membrane in that phosphatidyl glycerol and phosphatidyl ethanolamine increased with increasing BChl content, whereas phosphatidyl choline remained constant. Gorchein et al. (10) found that a doubling in lipid P per cell occurred in the adaptation of *R. spheroides* from aerobic to photosynthetic growth conditions and that the ornithine lipid content of pigmented cells was four times that of aerobic cells. Lascelles and Szilagyi (22) also reported that pigmented *R. sphaeroides* contained more phospholipid than did nonpigmented organisms. The rate of increase in phospholipid to increase in protein was 1.8 times higher in photosynthetically grown cells. All phospholipids increased, but phosphatidyl glycerol increased most rapidly, whereas phosphatidyl choline increased at the slowest rate. The presence of a higher lipid content associated with photosynthetic cells suggested an association between phospholipid synthesis and BChl production. The observation by Lascelles and Szilagyi (22) that phospholipid synthesis accompanied pigment formation raised questions concerning the dependence of BChl formation upon lipid synthesis. Therefore, it was of interest to determine whether BChl production required concurrent lipid synthesis.

In the absence of net phospholipid synthesis, C2 synthesized BChl at a slower rate than did supplemented cells. It appears that lipid synthesis is required for the normal rate of BChl production. Glycerol supplementation of deprived cells resulted in the resumption of BChl synthesis at the normal supplemented rate. It is of interest that cerulenin, a drug that inhibits fatty acid synthesis (9), also inhibited BChl synthesis.

Lascelles (19) isolated mutants of *R. spheroides* that are blocked in the synthesis of BChl, but accumulate pigments. One mutant, designated 2/73, accumulated magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester, whereas the mutant 2/21 accumulated the magnesium derivative of 2-devinyl-2-hydroxyethylphaeophorbid a. These pigments are produced under semianaerobic conditions and appear extracellularly when Tween 80 is present. In the absence of Tween 80, the pigments are retained within the cell and appear in the particulate fractions of cell-free extracts (19). To determine whether deprived cultures of C2 were synthesizing and accumulating or excreting magnesium pyrrole precursors, we measured BChl in cell-free fluid and in cellular extracts. These pigments absorb below 700 nm and would not interfere with the spectral determination of BChl at 775 nm. Neither Bchl nor precursor pigments were excreted into the medium by deprived or supplemented cells of C2.

Alternatively, the complete Bchl molecule may be synthesized at the normal rate in the deprived state, but degraded quite rapidly. However, when prelabeled cells were switched to cold medium, there was a slight increase in the number of counts incorporated by both deprived and supplemented cultures, indicating that a precursor pool may be labeling newly synthesized Bchl.

ALA, formed by the condensation of glycine and succinyl coenzyme A, is a precursor of BChl. The enzyme ALA synthase found in soluble extracts of *R. spheroides* and *R. rubrum* catalyzes the formation of ALA (15). This enzyme is under some kind of regulation, but the control of ALA synthase is still not understood. When the oxygen tension of a semianaerobic culture is increased by vigorous aeration, ALA formation stops and subsequently BChl synthesis ceases (14). During adaptation from aerobic to photosynthetic growth conditions, *R. spheroides* has an increased ability to form ALA (18), and the level of ALA synthase increases 10-fold (10).

Since BChl synthesis was reduced in the absence of net phospholipid synthesis, we looked for a corresponding decrease in ALA synthase activity. Soluble extracts prepared from semianaerobic and photosynthetically grown cells with and without glycerol continued to synthesize ALA. ALA synthase activity increased in deprived cells, indicating that BChl synthesis was not being shut off at the level of ALA synthase, but probably after this step. Our finding that the incorporation of ALA into BChl is inhibited in deprived cells is consistent with the regulatory site being later than ALA synthase.

Another enzyme, magnesium protoporphyrin-*S*-adenosylmethionine methyltransferase, appears involved in the regulation of BChl

synthesis. The formation of this enzyme is normally repressed in the presence of oxygen, but it is formed and active in aerobic cultures of a mutant of *R. spheroides* called TA-R, which was isolated by Lascelles and Wertlieb (23). The TA-R mutant forms BChl and carotenoids when grown aerobically in the dark. This mutant also had a lower level of reduced nicotinamide adenine dinucleotide oxidase activity as compared with the wild type under aerobic conditions, indicating that the electron transfer chain may be involved in O_2 repression of BChl synthesis (23). However, the respiration rate was normal. There is also indirect evidence that magnesium tetrapyrroles may regulate ALA synthase (21).

It has previously been shown that there is an obligatory coupling of chlorophyll synthesis and protein synthesis (18). Pigment synthesis is inhibited by amino acid deprivation or by the addition of inhibitors of protein or nucleic acid synthesis. When C2 is deprived of glycerol, protein synthesis continued in the absence of lipid synthesis, resulting in membranes with a higher protein content (unpublished data). The readdition of glycerol resulted in a resumption of pigment synthesis. BChl synthesis did not resume after the addition of both glycerol and chloramphenicol, indicating that simultaneous protein synthesis is necessary for pigment production.

Although carotenoid synthesis usually accompanies BChl formation, BChl can be synthesized in the absence of carotenoid synthesis and carotenoid production can occur without BChl formation. Mutants blocked in BChl synthesis that accumulate BChl precursors such as magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester and the magnesium derivative of 2-devinyl-2-hydroxyethyl phaeophorbid also make carotenoids (20).

Cohen-Bazire et al. (4) reported that the ratio of chlorophyll to carotenoid of *R. spheroides* is affected by the light intensity. The molar ratio of chlorophyll to carotenoid decreased as the light intensity increased. The major carotenoid present in semianaerobic cultures of C2 is pigment R, which is also known as spheroidenone (13). Since pigment Y and its hydroxy derivative disappear completely under semianaerobic conditions, only red carotenoids were measured spectrally. Spectral measurement of carotenoid synthesis indicates that deprived cells synthesize carotenoids at 30% of the rate of supplemented cells. The reduction in carotenoid synthesis could be due to the cessation of net lipid synthesis or could be a result of a decreased rate of BChl production. The cessation of phospholipid synthesis resulted in a reduction of both BChl and carotenoid synthesis to 30% of the supplemented rate. Therefore, the ratio of chlorophyll to carotenoid remained constant under deprived and supplemented conditions. This could be expected since semianaerobic conditions were maintained and there was no change in light intensity.

The results indicated that carotenoid synthe- and supplemented cells appears the same when measured by [^{14}C]acetate incorporation into carotenoid. This method is less accurate than is the spectral method, and one must account for precursor pools, turnover rates, and labeling of carotenoid derivatives, precursors, and degradation products that may migrate at the same rate as the completed carotenoid molecule.

The results indicated that carotenoid synthesis is affected by the cessation of net lipid synthesis. It is not known whether lipid synthesis exerts a direct regulatory control on carotenoid synthesis or whether the slowdown in carotenoid synthesis is a secondary effect of decreased BChl production. These results are similar to those of Ray et al. (35) in which they showed that carotenoid synthesis in an *S. aureus* glycerol auxotroph was reduced by glycerol removal. The incorporation of [^{14}C]acetate into nonpolar and polar carotenoids by deprived cultures of *S. aureus* was half the rate of supplemented cultures, indicating that lipid synthesis exerts control over carotenoid synthesis.

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