JOURNAL OF BACTERIOLOGY, June 1979, p. 788-798
0021-9193/79/06-0788/11$02.00/0

Vol. 138, No. 3

# Membranes of *Rhodopseudomonas sphaeroides*: Effect of Cerulenin on Assembly of Chromatophore Membrane

RICHARD M. BROGLIE AND ROBERT A. NIEDERMAN*

*Department of Microbiology, Bureau of Biological Research, Rutgers University, New Brunswick, New Jersey 08903*

Received for publication 19 March 1979

The effects of cerulenin were investigated in *Rhodopseudomonas sphaeroides* to elucidate further the mechanisms controlling the assembly of the chromatophore membrane. When this potent inhibitor of fatty acid biosynthesis was added to photosynthetically grown cultures, there was an immediate cessation of phospholipid, bacteriochlorophyll *a*, carotenoid, and ubiquinone formation. Concurrently, there was also a marked decrease in the rate of incorporation of protein into the chromatophore membrane. In contrast, only a small decrease in the rate of soluble and cell envelope protein synthesis was observed and, in chemotrophically grown cells, protein continued to be incorporated into both the cytoplasmic and outer membranes. The removal of δ-aminolaevulinate from mutant H-5 of *R. sphaeroides*, which requires this porphyrin precursor, was reexamined to determine whether cerulenin-induced cessation of chromatophore protein incorporation was due solely to blocked bacteriochlorophyll *a* synthesis. In the deprived H-5 cells, inhibition of [$^{35}$S]methionine incorporation into chromatophores was confined mainly to apoproteins of bacteriochlorophyll *a* complexes. Other minor chromatophore proteins continued to be inserted to a greater extent than in cerulenin-treated wild type where phospholipid synthesis had also ceased. These results indicate that the assembly of the chromatophore membrane is under strict regulatory control involving concomitant phospholipid, pigment, and protein syntheses.

During recent years, mechanisms involved in the assembly of biological membranes have become a focus of intense investigation. The photosynthetic apparatus of the facultatively photoheterotrophic bacterium *Rhodopseudomonas sphaeroides* provides a useful model system for such studies. Thin sections of cells grown under aerobic (chemotrophic) conditions reveal a typical gram-negative cell envelope with the cytoplasmic membrane closely apposed to the cell wall (7, 45). However, cells grown under conditions of reduced oxygen tension in either light or darkness (photoheterotrophic) elaborate an extensive system of intracytoplasmic membranes (termed the chromatophore membrane [39]) that is thought to arise by invagination of the cytoplasmic membrane (42). Mechanical disruption of cells grown under the latter conditions results in the release of a nearly uniform population of membrane vesicles (termed chromatophores) which can be easily purified to homogeneity (38, 39). Chromatophores have been shown to contain the photosynthetic apparatus of the cell (14, 24).

One approach to the study of membrane assembly involves uncoupling protein and phospholipid syntheses. This has been accomplished in bacterial systems by depriving glycerol-requiring auxotrophs of this polyol (2, 28, 35, 37, 47). Under such circumstances, phospholipid is no longer synthesized, and it is possible to study the incorporation of membrane proteins independent of concomitant phospholipid insertion (34, 36, 37). During cessation of phospholipid synthesis in a glycerol auxotroph of the related photosynthetic bacterium *Rhodopseudomonas capsulata*, protein synthesis continued for one doubling, whereas that of bacteriochlorophyll *a* (Bchl) and carotenoids was markedly inhibited (28). An association between the synthesis of phospholipid and photosynthetic pigments was demonstrated previously during chromatophore membrane induction in *R. sphaeroides* (30). In an effort to further elucidate these relations, the effect of the antibiotic cerulenin on the formation of the chromatophore membrane was investigated in detail. Cerulenin [(3*R*)-2,3-epoxy-4-oxo-7,10-dodecadienoyl amide (44)] is a potent inhibitor of fatty acid synthesis through inactivation of β-ketoacyl-acyl carrier protein synthetase (18). This ultimately results in the cessation of phospholipid formation. The effects of ceru-

lenin reported here suggest that the assembly of the chromatophore membrane is under strict regulatory control involving concomitant phospholipid, pigment, and protein syntheses.

## MATERIALS AND METHODS

**Growth of organism.** *R. sphaeroides* NCIB 8253 was grown photoheterotrophically in the medium of Cohen-Bazire et al. (15) at 30°C with a constant illumination of 1,830 lx. Growth was followed by monitoring the increase in optical density at 680 and 850 nm with a Beckman DU spectrophotometer (1-cm light path) equipped with a red-sensitive phototube. Cells were grown aerobically in the same medium on a Gyrotory shaker (model G-25; New Brunswick Scientific Co., New Brunswick, N.J.) at 350 rpm.

*R. sphaeroides* H-5 (provided by J. Takemoto) was grown photosynthetically as described for the wild type except that 1.0 mM δ-aminolaevulinic acid (ALA) was included in the growth medium.

**Isotope labeling procedures.** Photosynthetically grown cultures were maintained in an atmosphere of 95% $N_2$–5% $CO_2$ until an optical density of 0.6 to 0.7 at 680 nm was obtained. Cerulenin (20 μg/ml) was added to one half of the culture, and growth was allowed to continue for 1 h. Cultures were then labeled with $^{32}P_i$ (0.5 μCi/ml) and L-[$^3$H]phenylalanine (0.75 μCi and 15 μg/ml) or L-[$^{35}$S]methionine (0.4 μCi and 10 μg/ml) and immediately aliquoted into 50-ml narrow-necked test tubes filled to capacity. At 0.5, 1, 2, 3, 5, and 7 h, 45 ml each of control and cerulenin-treated cells were diluted into 2 volumes of crushed ice to prevent further incorporation of radioactivity. Cells were collected by centrifugation at 4°C in a Sorvall GSA rotor at 10,000 rpm for 10 min (16,300 × *g*). The harvested cells were washed twice in 1.0 mM Tris buffer, pH 7.5. Experiments to determine the effect of cerulenin on chemotrophic cells were performed as described above, except that maximal aeration was maintained during cell growth. Harvested aerobic cells were washed twice in 0.01 M Tris, pH 8.1.

For studies in which the synthesis of Bchl was inhibited, H-5 cultures were grown as above until an optical density of 0.6 to 0.7 at 680 nm was obtained. Cells were then aseptically collected by centrifugation, washed with sterile saline, and resuspended in fresh medium with or without ALA supplementation. L-[$^3$H]phenylalanine or L-[$^{34}$S]methionine and $^{32}P_i$ were used to monitor protein and phospholipid syntheses, respectively.

$^{32}P_i$ was purchased from ICN, Irvine, Calif., and L-[$^3$H]phenylalanine and L-[$^{35}$S]methionine were purchased from New England Nuclear Corp., Boston, Mass. Cerulenin was obtained from Makor Chemical Co., Jerusalem, Israel.

**Membrane isolation.** Phototrophic cells were disrupted by passage through a French pressure cell (American Instrument Co., Inc., Silver Spring, Md.) at 18,000 lb/in$^2$. Chromatophore and cell envelope fractions were isolated by differential and rate-zone sedimentation in a Beckman L2-65B ultracentrifuge as described previously (40), except that a Beckman SW40 Ti rotor was employed.

Cell-free extracts of aerobically grown cells were prepared by the EDTA-lysozyme-Brij lysis procedure described by Collins and Niederman (16). Crude membranes were isolated by centrifugation of the cell-free extract for 75 min at 166,500 × $g_{av}$ in a Beckman 50 Ti rotor. They were washed twice in 0.02 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)–0.01 M EDTA, pH 7.5. After the washing procedure, the crude membrane fraction was layered onto a gradient prepared with 20, 40, and 60% (wt/wt) sucrose (19) in the same buffer and centrifuged for 4 h at 234,000 × $g_{av}$ in a Beckman SW50.1 swinging-bucket rotor. Assays of succinate dehydrogenase (succinate: phenazine methosulfate oxidoreductase [EC 1.3.99.1]) activity (16) and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of the resolved cytoplasmic and outer membranes indicated that the separation was essentially complete (data not shown).

**Chemical analyses.** Protein was determined by the method of Lowry et al. (32), using bovine serum albumin (crystalline; Miles Laboratories, Inc., Elkhart, Ind.) as the standard. Membranes were treated with 0.5 N NaOH at 40°C for 60 min before the initiation of the color reaction. Total phosphorous of purified chromatophores was determined by the method of Chen et al. (9) on samples that were wet-ashed by the method of LePage (31). Bchl and carotenoid levels were determined by extraction with acetone-methanol (7:2, vol/vol) as described by Cohen-Bazire et al. (15). An extinction coefficient of 82 $mg^{-1}$ $cm^{-1}$ (11) at 770 nm was used for Bchl. Carotenoid absorbance was monitored at 456 nm for the yellow carotenoid (spheroidene) and 510 nm for the red carotenoid (spheroidenone). Levels of the carotenoids were calculated with a computer program, using the equations of Cohen-Bazire et al. (15). Ubiquinone was extracted from whole cells by the procedure described by Takamiya and Takamiya (51). The levels of ubiquinone in the extracts (0.1 ml) were assayed spectrophotometrically (17) after the addition of ethyl cyanoacetate (0.8 ml; Aldrich Chemical Co., Milwaukee, Wis.) and 5% ethanolic KOH (0.1 ml). The absorbance of the cyanoacetate derivative of ubiquinone ($\lambda_{max}$ = 620 nm) was recorded at 5 min after the addition of the reagents. Ubiquinone-10 (Sigma Chemical Co., St. Louis, Mo.) was employed as a standard.

**Cell fractionation.** Whole cells were precipitated from the media with ice-cold 10% trichloroacetic acid (1:1, vol/vol) for 30 min. The pellet was collected by centrifugation, washed with ice-cold 5% trichloroacetic acid, and recentrifuged. Lipids were extracted from the acid-insoluble material by the method of Ames (1). To insure quantitative extraction of lipids, trichloroacetic acid pellets were routinely extracted at least twice by this procedure. The washed chloroform-methanol extracts were pooled, evaporated to dryness under a stream of nitrogen, and saved for determination of radioactivity. Nucleic acids were extracted from the organic solvent-insoluble material with 5% trichloroacetic acid at 95°C for 60 min. Hot trichloroacetic acid precipitates were collected by centrifugation and washed with diethyl ether to remove excess trichloroacetic acid.

**Radioactivity measurements.** Radioactivity was determined on a Beckman LS-230 liquid scintillation counter. All samples were counted in 10 ml of a tolu-

ene-based scintillation cocktail containing 8 g of PPO (2,5-diphenyloxazole) and 0.2 g of dimethyl-POPOP {1,4-bis-[2]-(4-*methyl*-5-phenyloxazoyl)benzene} per liter. Aqueous samples were made miscible with the cocktail by the addition of NCS (Amersham Corp., Arlington Heights, Ill.) (NCS/sample ratio, 6:1). Trichloroacetic acid precipitates were solubilized in NCS at 50°C for 1 h. Double-labeled membrane preparations were solubilized in NCS and counted without further chemical fractionation. This was shown to be unnecessary since >99% of [$^3$H]phenylalanine in purified chromatophores was incorporated into hot acid-precipitable material, and at least 95% of the total $^{32}$P counts were extracted by chloroform-methanol; the latter suggested that the isolated membranes were essentially free of contamination by nucleic acid (unpublished data). Pigmented samples were decolorized before counting by the addition of 0.5 ml of a saturated solution of benzoyl peroxide in toluene.

Radioautography of labeled proteins was performed by impregnating SDS-polyacrylamide gels with PPO by the procedure of Bonner and Laskey (6). The dried gel was exposed to X-ray film (du Pont Cronex-2DC; E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) and maintained at −70°C for 48 h.

**Electrophoresis.** Membrane protein composition was analyzed by SDS-polyacrylamide gel electrophoresis, employing the discontinuous buffer system described by Laemmli and Favre (29). Electrophoresis was carried out in a Hoefer vertical slab apparatus (model SE500; Hoefer Scientific Instruments, Inc., San Francisco, Calif.). Maximal resolution of the lower-molecular-weight membrane polypeptides was achieved with a separating gel (9 to 10 cm) prepared as a linear-concentration gradient of 10 to 14% acrylamide accompanied by a 5 to 20% sucrose gradient. The stacking gel (1 to 2 cm) consisted of 6% acrylamide. In both cases the ratio of polyacrylamide to *N,N'*-methylenebisacrylamide was 30:0.8. Polymerization of the gradient gel was initiated chemically with 0.02% *N,N,N',N'*-tetramethylethylenediamine and 0.2% $(NH_4)_2S_2O_8$. Polymerization was complete within 60 min after the addition of the catalysts.

Membrane samples (85 μg of protein) were prepared for electrophoresis in a mixture containing the following in final concentrations: 62.5 mM Tris-hydrochloride (pH 6.8), 2.0% SDS, 50 mM dithiothreitol, 10% glycerol, and 0.001% bromophenol blue. Solubilization was achieved by heating in a boiling-water bath for 60 s.

Electrophoresis was performed initially at 60 V and increased to 120 V after penetration of the tracking dye into the separating gel. Gels were fixed overnight in a solution containing 25% isopropanol and 10% glacial acetic acid and stained for 3 to 5 h with Coomassie brilliant blue R (0.2% in 25% isopropanol–10% glacial acetic acid). Gels were destained by diffusion at 45°C against repeated changes of fixing solution.

## RESULTS

**Effect of cerulenin concentration on cellular protein and phospholipid synthesis.** A necessary prerequisite for studying membrane assembly by the uncoupling of phospholipid synthesis from that of protein is a suitable inhibitor concentration which allows the formation of protein to continue in the absence of any appreciable additional lipid. In this regard, exponentially growing phototrophic cultures of *R. sphaeroides* were treated with various concentrations of cerulenin for 1 h, followed by labeling with [$^3$H]phenylalanine and $^{32}P_i$ for 90 min. Cellular growth was halted by precipitation of the cells with an equal volume of 10% trichloroacetic acid, and the amount of radioactivity incorporated into total phospholipid and protein was determined as described above. The results of this experiment are shown in Fig. 1. For subsequent experiments, a cerulenin concentration of 20 μg/ml was chosen. This concentration of antibiotic caused a greater than 95% inhibition of phospholipid synthesis, whereas the rate of protein synthesis remained at 80% of the control level.

**Effect of cerulenin on the phototrophic growth of *R. sphaeroides*.** The concentration of cerulenin (20 μg/ml) employed for these experiments, although sufficient to effectively inhibit phospholipid synthesis, did not result in a complete cessation of growth (Fig. 2). The generation time of the cerulenin-treated culture was 8 h, whereas that of the untreated control culture was 5.3 h. Cellular morphology, as examined by phase-contrast microscopy, appeared normal in the cerulenin-treated culture until 7 to 8 h of treatment. Such prolonged growth in the absence of phospholipid synthesis resulted in an increase in the number of cells occurring in chains, presumably caused by incomplete cell division. It can also be seen (Fig. 2) that the

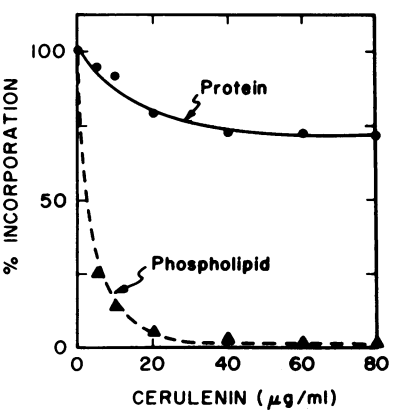


FIG. 1. *Effect of cerulenin concentration on cellular protein and phospholipid syntheses measured by the incorporation of [$^3$H]phenylalanine and $^{32}P_i$ into the hot trichloroacetic acid- or organic solvent-soluble fractions, respectively. Values are expressed as the percentage of total counts incorporated into a control culture without cerulenin. Experimental parameters are described in the text.*

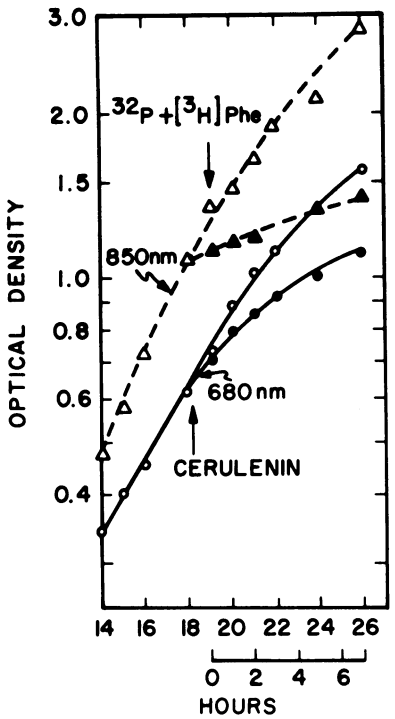


FIG. 2. *Effect of cerulenin on the growth of R. sphaeroides. Culture turbidity was monitored at 680 nm, whereas 850 nm represents the in vivo absorption maximum of Bchl-protein complexes. Open symbols are controls; closed symbols indicate cultures grown in the presence of cerulenin. Arrows indicate the time of addition of antibiotic and radiolabeled tracers. Growth conditions are as described in the text.*

cerulenin-treated culture exhibited a marked decrease in absorbance at 850 nm (the in vivo absorbance maximum of Bchl-protein complexes). This was quantified further by acetone-methanol extractions of whole cells (Fig. 3). It can be seen that in the presence of cerulenin, the synthesis of Bchl, as well as those of spheroidenone and spheroidene, are inhibited by greater than 90% in each case (Fig. 3). Klein and Mindich (28) have also observed a similar cessation of photopigment synthesis in the absence of phospholipid synthesis in a glycerol auxotroph of a related photosynthetic bacterium, *R. capsulata*.

One possible explanation for the observed inhibition of Bchl and carotenoid syntheses in the presence of cerulenin could be the effect of this antibiotic on the formation of isoprenoid-containing compounds (see Fig. 10), since cerulenin has been shown to inhibit the incorporation of [$^{14}$C]acetyl coenzyme A (acetyl-CoA) into the non-saponifiable fraction in a cell-free system of yeast (41, 44). Thus, in vivo, cerulenin would be expected to inhibit the formation of these compounds, which include carotenoids (27), the phytol group of Bchl (5), and ubiquinone (54). To further test this possibility, the cellular level of ubiquinone was determined in cultures grown photosynthetically in the presence of cerulenin (20 μg/ml). These results (Fig. 3) clearly demonstrate that cerulenin treatment also results in the cessation of ubiquinone biosynthesis.

**Effect of cerulenin on cellular protein and phospholipid syntheses.** The effect of cerulenin on the incorporation of [$^{3}$H]phenylalanine and $^{32}P_i$ into cellular protein and phospholipid, respectively, was examined by labeling phototrophic cells in the presence and absence of cerulenin. Figure 4 shows that for at least 8 h after the addition of the antibiotic, protein synthesis continued (albeit at a reduced rate) in the absence of further phospholipid synthesis. This is consistent with the results obtained by others with the antibiotic cerulenin (25), as well as those obtained with glycerol auxotrophs under conditions of glycerol starvation (2, 28, 35, 47).

**Effect of cerulenin on membrane assembly.** Since cellular protein synthesis was shown to continue at a reduced rate in the presence of cerulenin, it was necessary to distinguish whether this occurred as a result of the decreased growth rate or, alternatively, reflected the specific inhibition of either soluble or membrane protein synthesis. Much of the synthesis of soluble protein components (Fig. 5b) continued when phototrophically grown cells were treated with cerulenin.

It was expected from the results of others (10,

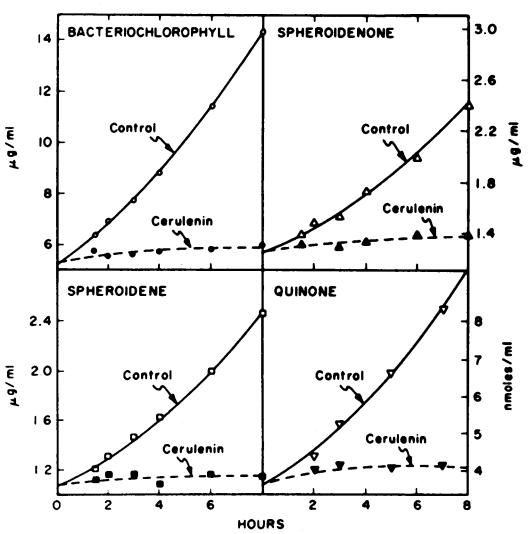


FIG. 3. *Effect of cerulenin on total cellular Bchl, spheroidene, spheroidenone, and ubiquinone biosyntheses. All measurements were performed as described in the text.*

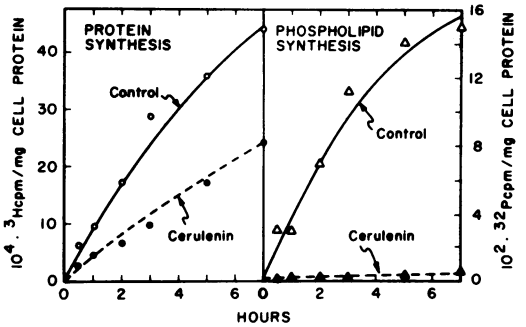


FIG. 4. *Incorporation of [$^3$H]phenylalanine or $^{32}P_i$ into cellular protein and phospholipid, respectively, in cerulenin-treated and control cultures.*

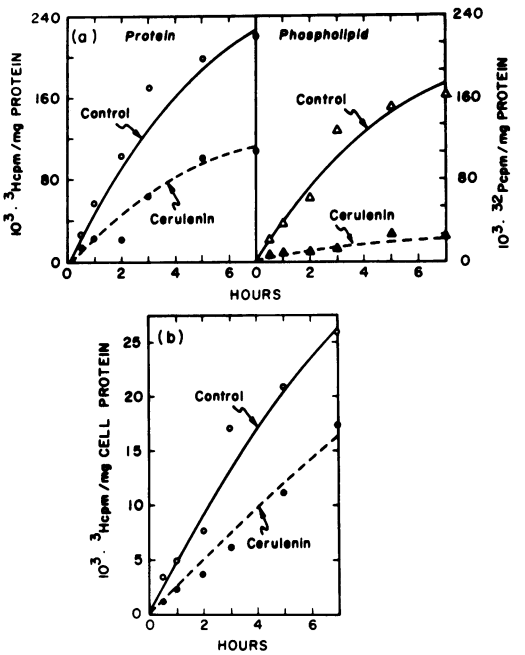


FIG. 5. *(a) Incorporation of protein and phospholipid into the cell envelope fraction isolated from cells grown photosynthetically in the presence or absence of cerulenin. (b) Rate of soluble protein synthesis in control and cerulenin-treated cultures.*

34, 36, 46) that the incorporation of membrane proteins should continue in the absence of phospholipid synthesis. Cerulenin treatment confirmed this with both the cytoplasmic and outer membranes of aerobically grown *R. sphaeroides* (Fig. 6), as well as with the envelope fraction obtained from photosynthetically grown cells (Fig. 5a). In the chromatophore fraction, however, an essentially complete inhibition of protein incorporation was observed in cells treated with cerulenin (Fig. 7).

In a study with *R. sphaeroides* mutants blocked at various stages in Bchl synthesis, it was shown that the completed Bchl molecule is necessary for the insertion of chromatophore-specific proteins into the membrane (52). It therefore seemed possible that the cerulenin-induced cessation of chromatophore protein assembly was due to the ability of this antibiotic to block Bchl synthesis. To test this possibility, the effect of specific inhibition of Bchl synthesis on the assembly of the chromatophore membrane in the ALA-requiring mutant H-5 was reexamined and compared with the cerulenin treatment of the wild type. During ALA depri-

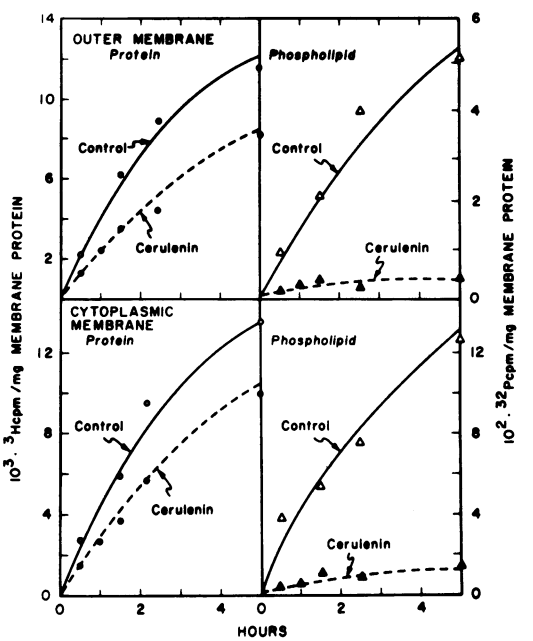


FIG. 6. *Effect of cerulenin on the incorporation of protein and phospholipid into the cytoplasmic and outer membranes isolated from aerobically grown cells.*

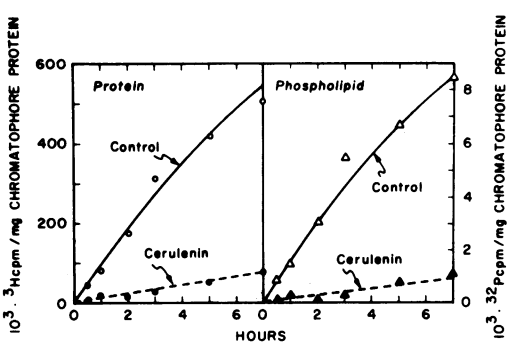


FIG. 7. *Incorporation of protein and phospholipid into the chromatophore membrane of control and cerulenin-treated cells.*

vation of the mutant, in confirmation of a previous result (52), the incorporation of [$^3$H]phenylalanine into the chromatophore membrane was diminished by 55%, whereas that of phospholipid remained unaffected. This is in contrast to the effect of cerulenin on chromatophore membrane assembly in the wild type, which, as noted above, results in a much greater inhibition of membrane protein incorporation, as well as an inhibition of phospholipid and photopigment syntheses.

SDS-polyacrylamide gel electrophoresis and fluorographic analysis of samples isolated from cells labeled with [$^{35}$S]methionine at the time of ALA removal (mutant H-5) or after cerulenin treatment (wild type) were employed to study the appearance of newly synthesized proteins in the chromatophore membrane. The Coomassie brilliant blue staining profiles (Fig. 8, upper gels) of membrane samples from both the inhibited wild type and the ALA-deprived mutant indicate no gross changes in polypeptide composition. In the cerulenin-inhibited cells, however, the fluororadiogram confirms that the inhibition of chromatophore-protein incorporation is essentially complete (Fig. 8A, lower left), concomitant with cessation of both pigment and phospholipid syntheses (Fig. 7). As reported previously for the ALA-deprived strain (52), inhibition is confined mainly to established apoproteins of the Bchl complexes after the specific blockage of Bchl synthesis (Fig. 8B, lower right). In contrast, scans of the fluororadiograms (Fig. 9) indicate that minor chromatophore polypeptide components continue to be inserted into the chromatophore membrane of ALA-deprived H-5 to an extent greater than that in the cerulenin-treated wild type in which phospholipid synthesis had also ceased. These components were enriched in the fluororadiography profile of the chromatophores from the ALA-depleted cells. With regard to the polypeptide components of the Bchl complexes, those of the photochemical reaction center (RC) and some of those associated with light-harvesting (LH) Bchl are not incorporated into the chromatophore membrane of the deprived mutant; other apparent LH Bchl-associated components were incorporated but in reduced amounts (Fig. 8A). The gradient gel procedure has resulted in separation of the LH Bchl complexes into the multiple-polypeptide components recently reported for *R. capsulata* (20, 49); those believed to be associated with the accessory LH Bchl complex with an absorption maximum at 850 nm (B850) continue to be incorporated at a reduced rate. The polypeptide component thought to be associated with the RC-associated LH Bchl complex with a maximum near 875 nm (B875) is not incorporated into the membrane.

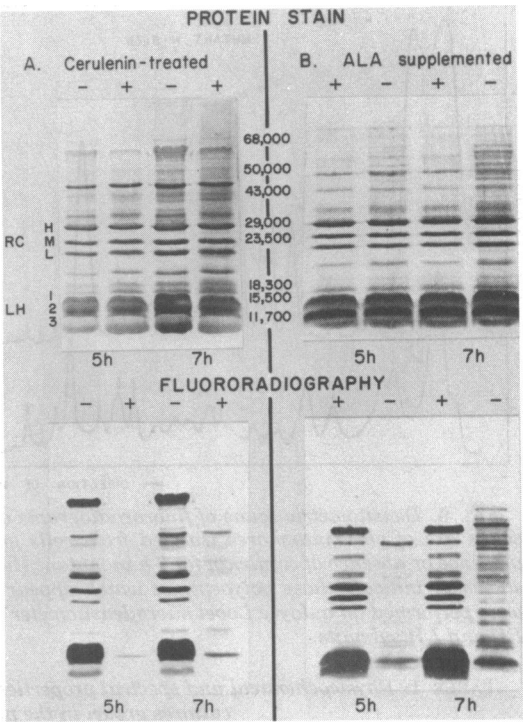


FIG. 8. *SDS-polyacrylamide gel electrophoresis of purified chromatophores isolated from cells labeled with [$^{35}$S]methionine after (A) cerulenin treatment of wild-type R. sphaeroides and (B) ALA deprivation of the auxotrophic strain. The polypeptide pattern was obtained by Coomassie brilliant blue staining (upper gels); whereas the incorporation of label was detected by fluororadiography (lower gels). Membranes shown were isolated from the cells after 5 and 7 h of treatment. The control is represented by the left lane of each pair of samples. $RC_{H,M,\text{and}\,L}$ refer to the RC polypeptide subunits (13); $LH_{1\,to\,3}$ denote apparent LH Bchl-associated polypeptides (12, 20, 21, 49). Molecular weights obtained with protein standards are listed in upper gels.*

As a result of continued phospholipid insertion during ALA deprivation and the absence of newly inserted apoprotein components of the Bchl complexes, changes in the protein/phospholipid ratio (and consequently the specific buoyant density) would be expected, as these protein components can comprise up to 60% of total chromatophore protein (12, 21). As shown in Table 1, however, both the protein/phospholipid ratio and the buoyant density, as determined by isopycnic centrifugation in CsCl gradients (16), remain virtually unchanged after cessation of Bchl synthesis. The relative decrease in specific Bchl content as well as in B850 and constancy of the protein/phospholipid ratio

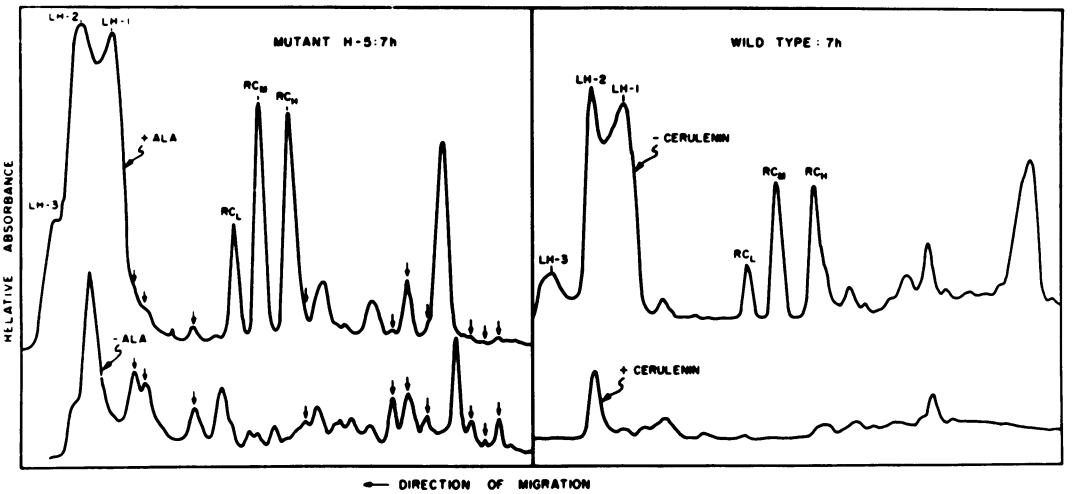


FIG. 9. *Densitometric scans of fluororadiograms obtained from polyacrylamide gels shown in Fig. 8. The scans are of chromatophores isolated from cells grown with or without ALA supplementation or in the presence or absence of cerulenin for 7 h in mutant H-5 and wild-type R. sphaeroides, respectively. Arrows are shown to indicate those polypeptides which appear enriched in the ALA-depleted chromatophores. Scans were performed on a Joyce Lobel microdensitometer (model 3CS). See the legend to Fig. 8 for designations of RC and LH subunits.*

TABLE 1. *Physicochemical and spectral properties of chromatophores isolated from R. sphaeroides H-5 cultures grown in the presence or absence of ALA*

| Chromatophore sample (h) | Specific Bchl[a] | | Protein/phospholipid | | ρ 25°C[b] | | B875/B850[c] | |
|---|---|---|---|---|---|---|---|---|
| | +ALA | −ALA | +ALA | −ALA | +ALA | −ALA | +ALA | −ALA |
| 0.5 | 63.6 | 65.1 | 2.11 | 2.17 | 1.19 | 1.19 | 0.75 | 0.75 |
| 1.0 | 65.7 | 67.7 | 2.16 | 2.15 | —[d] | —[d] | 0.66 | 0.67 |
| 4.0 | 67.5 | 63.4 | 2.19 | 2.07 | 1.185 | 1.185 | 0.64 | 0.72 |
| 6.0 | 70.2 | 59.9 | 2.16 | 2.07 | 1.19 | 1.19 | 0.63 | 0.71 |

[a] Micrograms of Bchl per milligram of protein.
[b] Determined as described previously (16).
[c] Spectral analysis performed as described previously (40).
[d] —, Values not determined.

and specific density suggest that inhibition of Bchl synthesis does not alter the incorporation of many of the proteins into the bilayer concurrent with phospholipid. However, in the absence of Bchl synthesis, the newly inserted proteins are mainly confined to those components normally found as minor species in chromatophores from supplemented cells (Fig. 8B, lower right). In contrast, cerulenin, presumably through the inhibition of both phospholipid and photopigment syntheses, appeared to prevent much of the incorporation of all chromatophore proteins.

## DISCUSSION

Cerulenin, a potent inhibitor of fatty acid biosynthesis via the specific inactivation of β-ketoacyl-acyl carrier protein synthetase (18), causes both phospholipid and photopigment syntheses to cease in phototrophically grown *R. sphaeroides*. Concurrently, there is also a specific and marked decrease in the rate of incorporation of newly synthesized protein into the chromatophore membrane. In contrast, when Bchl synthesis is inhibited, both phospholipid and protein, with the exception of pigment-associated apoproteins (52), continue to be incorporated into chromatophores; other minor chromatophore protein components appear enriched.

An association between phospholipid and photopigment syntheses was observed by Lascelles and Szilagyi (30) during induction of chromatophore membrane formation. It has been suggested (28) from investigations of an *R. capsulata* glycerol auxotroph that this association may reflect an actual dependence of Bchl synthesis on that of phospholipid. This proposal was supported by the addition of cerulenin to wild-type *R. capsulata*, which resulted in the concomitant inhibition of both Bchl and phos-

pholipid syntheses. However, this apparent dependence of Bchl on phospholipid synthesis would also be observed if cerulenin inhibited the formation of β-hydroxy-β-methylglutaryl-CoA (HMG-CoA), a precursor required for the synthesis of the phytol moiety of the Bchl molecule (Fig. 10). In support of this hypothesis was the inhibition by this antibiotic of both carotenoid and ubiquinone formations, other products of the isoprenoid biosynthetic pathway. Thus, the cerulenin-induced inhibition of Bchl synthesis may be due to the absence of HMG-CoA formation rather than a direct consequence of phospholipid cessation (Fig. 10). There is at least one report indicating that cerulenin inhibits HMG-CoA synthetase in yeast (43). A further test of this hypothesis would require the detection of bacteriochlorophyllide *a* accumulation in cerulenin-treated cells as well as an examination of the effect of cerulenin on *R. sphaeroides* HMG-CoA synthetase in vitro. These experiments are currently in progress.

Since several aspects of chromatophore formation are perturbed by cerulenin, it could be argued that the observed effect on Bchl synthesis might be caused by the cerulenin-induced inhibition of ubiquinone, carotenoid, or even chromatophore protein synthesis; however, neither the proposed biosynthetic pathway for isoprenoid-containing components (Fig. 10) nor the

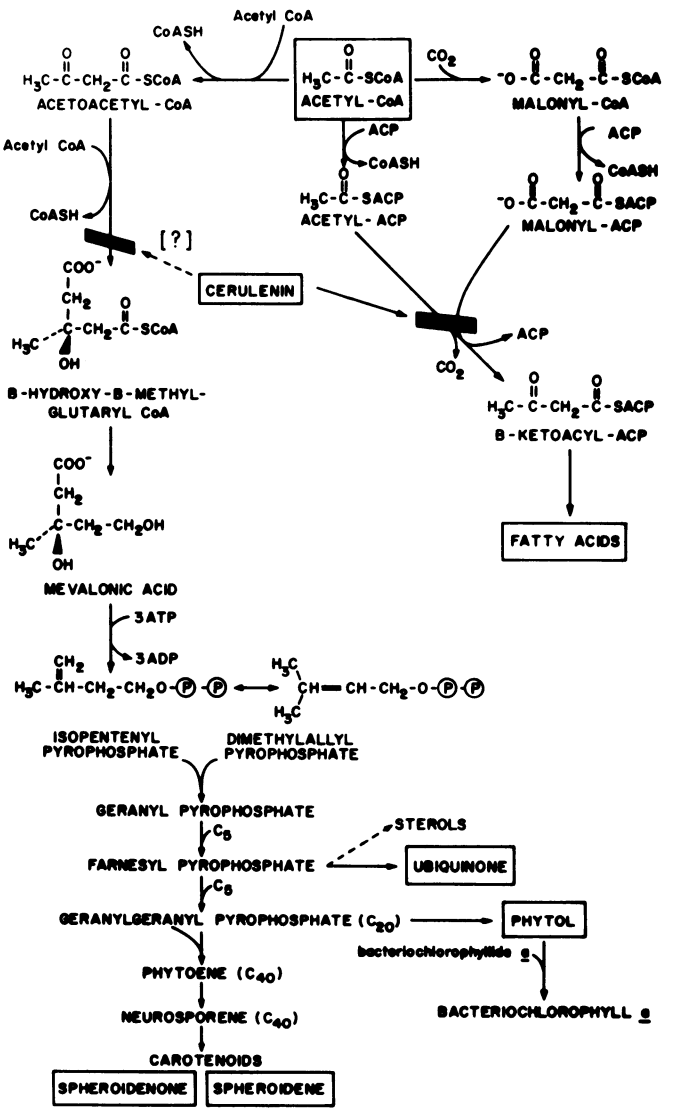


FIG. 10. *Proposed sites of action of cerulenin on fatty acid and isoprenoid biosyntheses in R. sphaeroides.*

finding that Bchl synthesis occurs (50) in a mutant lacking the RC polypeptide subunits (13) is in accord with these possibilities. Regarding the inhibition of Bchl synthesis in the *R. capsulata* glycerol auxotroph (28), it is possible that the accumulation of free fatty acids in the cell could affect the synthesis of compounds derived from the condensation of isoprenoid units (e.g., as in the case of cerulenin). Indeed, exogenously supplied fatty acids have been shown to result in a reduction of sterol biosynthesis in yeast (26). Such a regulatory effect may also explain the inhibition of vitamin K and carotenoid biosyntheses observed during glycerol deprivation of a *Staphylococcus aureus* glycerol auxotroph, which also accumulates free fatty acids under these conditions (47).

The results presented in this paper indicate that the assembly of the chromatophore membrane is under strict regulatory control, requiring the concomitant synthesis of phospholipid, pigments, and proteins. Such a regulatory mechanism can be envisioned as occurring at the level of membrane assembly or, alternatively, at the level of protein synthesis (i.e., transcription or translation). It seems likely, however, from our results and those previously reported (52) that both mechanisms govern the assembly of the chromatophore membrane in *R. sphaeroides*. The obligate coupling between the synthesis of Bchl and the appearance of Bchl-associated proteins in the membrane has already been established (52). This control may now be extended to include a requirement for insertion of phospholipid moieties into the membrane, without which the incorporation of additional protein is eventually curtailed. Although in synchronously dividing *R. sphaeroides* phospholipids are incorporated discontinuously into the chromatophore membrane (33) while protein is continuously inserted (23), the synthesis of the membrane also appears to be controlled ultimately by phospholipid biosynthesis through changes in membrane fluidity (see below). On the other hand, continuous phospholipid synthesis has been shown to be required for the assembly of cytoplasmically derived mitochondrial proteins in yeast (48) and the specific insertion of the f1 bacteriophage coat protein into the cytoplasmic membrane of *Escherichia coli* (8). In contrast, others have shown that the incorporation of membrane protein components can continue in the absence of phospholipid (10, 34, 46).

Such a coupling of phospholipid and protein insertion could indicate that a fluid hydrocarbon environment, provided by phospholipid molecules, is necessary to accept additional hydrophobic protein species. Alternatively, protein and phospholipid components may enter the growing membrane simultaneously, each dependent upon the other. This, however, is not likely since the bulk of the phospholipid-synthesizing enzymes have been shown to be integral membrane components of the bacterial cytoplasmic membrane (3, 53). Support for the former hypothesis comes from measurements of chromatophore membrane fluidity, employing the fluorescent probe $\alpha$-parinaric acid (22). These results indicate that the presence of protein in the chromatophore membrane imparts a two- to threefold-greater restriction on the mobility of the fatty acyl chains than that observed with extracted chromatophore phospholipids. It has also been shown that chromatophores contain an unusually high percentage of phospholipid that is immobilized by protein, especially under conditions of lower light intensity (4), thus reducing the amount of lipid in the bulk fluid phase. Others have noted that upon glycerol deprivation of an *E. coli* glycerol auxotroph, membrane synthesis is reduced to 60% of the control rate (34). This has been attributed to cellular control mechanisms which prevent protein saturation of lipid-depleted membranes. In this respect, the results reported here suggest that the nature of the cytoplasmic and outer membrane bilayers of aerobically grown *R. sphaeroides* are such that they are capable of accepting greater amounts of protein than is normally present under physiological conditions, whereas the chromatophore membrane is not.

## ACKNOWLEDGMENTS

This work was supported by National Science Foundation grants PCM74-18151 and PCM76-24142, Public Health Service grant GM20367-03 from the National Institute of General Medical Sciences, and the Charles and Johanna Busch Memorial Fund Award to the Rutgers Bureau of Biological Research. R.M.B. is the recipient of a Busch Predoctoral Fellowship Award, and R.A.N. is supported by Public Health Service Research Career Development Award GM00093-04 from the National Institute of General Medical Sciences.

We thank Janet C. Onishi for useful discussions and Margaret Lai for scans of the fluororadiograms.

## LITERATURE CITED

1. **Ames, G. F.** 1968. Lipids of *Salmonella typhimurium* and *Escherichia coli*: structure and metabolism. J. Bacteriol. **95**:833–843.
2. **Bell, R. M.** 1974. Mutants of *Escherichia coli* defective in membrane phospholipid synthesis: macromolecular synthesis in an sn-glycerol 3-phosphate acyltransferase $K_m$ mutant. J. Bacteriol. **117**:1065–1076.
3. **Bell, R. M., R. D. Mavis, M. J. Osborn, and P. R. Vagelos.** 1971. Enzymes of phospholipid metabolism: localization in the cytoplasmic and outer membrane of the cell envelope of *Escherichia coli* and *Salmonella typhimurium*. Biochim. Biophys. Acta **249**:628–635.
4. **Birrell, G. B., W. R. Sistrom, and O. H. Griffith.** 1978. Lipid-protein associations in chromatophores from the photosynthetic bacterium *Rhodopseudomonas sphaeroides*. Biochemistry **17**:3768–3773.
5. **Bogorad, L.** 1976. Chlorophyll biosynthesis, p. 64–148. *In* T. W. Goodwin (ed.), Chemistry and biochemistry of

plant pigments. Academic Press Inc., New York.
6. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83–88.
7. **Brown, A. E., F. A. Eiserling, and J. Lascelles.** 1972. Bacteriochlorophyll synthesis and the ultrastructure of wild type and mutant strains of *Rhodopseudomonas sphaeroides*. Plant Physiol. **50**:743–746.
8. **Chamberlain, B. K., and R. E. Webster.** 1976. Lipid-protein interactions in *Escherichia coli*. Membrane-associated f1 bacteriophage coat protein and phospholipid metabolism. J. Biol. Chem. **251**:7739–7745.
9. **Chen, P. S., T. Y. Toribara, and H. Warner.** 1956. Microdetermination of phosphorous. Anal. Chem. **28**: 1756–1758.
10. **Chopra, I.** 1975. Induction of tetracycline resistance in *Staphylococcus aureus* in the absence of lipid synthesis. J. Gen. Microbiol. **91**:433–436.
11. **Clayton, R. K.** 1963. Toward the isolation of a photochemical reaction center in *Rhodopseudomonas sphaeroides*. Biochim. Biophys. Acta **75**:312–323.
12. **Clayton, R. K., and B. J. Clayton.** 1972. Relations between pigments and proteins in the photosynthetic membranes of *Rhodopseudomonas sphaeroides*. Biochim. Biophys. Acta **283**:492–504.
13. **Clayton, R. K., and R. Haselkorn.** 1972. Protein components of bacterial photosynthetic membranes. J. Mol. Biol. **68**:97–105.
14. **Cohen-Bazire, G., and R. Kunisawa.** 1960. Some observations on the synthesis and function of the photosynthetic apparatus in *Rhodospirillum rubrum*. Proc. Natl. Acad. Sci. U.S.A. **46**:1543–1556.
15. **Cohen-Bazire, G., W. R. Sistrom, and R. Y. Stanier.** 1957. Kinetic studies of pigment synthesis by non-sulfur purple bacteria. J. Cell. Comp. Physiol. **49**:25–68.
16. **Collins, M. L. P., and R. A. Niederman.** 1976. Membranes of *Rhodospirillum rubrum*: isolation and physicochemical properties of membranes from aerobically grown cells. J. Bacteriol. **126**:1316–1325.
17. **Crane, F. L., and R. Barr.** 1971. Determination of ubiquinone. Methods Enzymol. **18C**:137–165.
18. **D'Agnolo, G., I. S. Rosenfeld, J. Awaya, S. Ōmura, and P. R. Vagelos.** 1973. Inhibition of fatty acid synthesis by the antibiotic cerulenin. Specific inactivation of β-ketoacyl-acyl carrier protein synthetase. Biochim. Biophys. Acta **326**:155–166.
19. **Ding, D. H., and S. Kaplan.** 1976. Separation of inner and outer membranes of *Rhodopseudomonas sphaeroides*. Prep. Biochem. **6**:61–79.
20. **Feick, R., and G. Drews.** 1978. Isolation and characterization of light harvesting bacteriochlorophyll·protein complexes from *Rhodopseudomonas capsulata*. Biochim. Biophys. Acta **501**:499–513.
21. **Fraker, P. J., and S. Kaplan.** 1972. Isolation of a bacteriochlorophyll-containing protein from *Rhodopseudomonas sphaeroides*. J. Biol. Chem. **247**:2732–2737.
22. **Fraley, R. T., D. M. Jameson, and S. Kaplan.** 1978. The use of the fluorescent probe α-parinaric acid to determine the physical state of the intracytoplasmic membranes of the photosynthetic bacterium, *Rhodopseudomonas sphaeroides*. Biochim. Biophys. Acta **511**:52–69.
23. **Fraley, R. T., D. R. Lueking, and S. Kaplan.** 1978. Intracytoplasmic membrane synthesis in synchronous cell populations of *Rhodopseudomonas sphaeroides*. Polypeptide insertion into growing membranes. J. Biol. Chem. **253**:458–464.
24. **Frenkel, A. W., and D. D. Hickman.** 1959. Structure and photochemical activity of chlorophyll-containing particles from *Rhodospirillum rubrum*. J. Biophys. Biochem. Cytol. **6**:285–290.
25. **Goldberg, I., J. R. Walker, and K. Bloch.** 1973. Inhibition of lipid synthesis in *Escherichia coli* cells by the antibiotic cerulenin. Antimicrob. Agents Chemother. **3**: 549–554.
26. **Greenspan, M. D., R. C. Mackow, and S. Ōmura.** 1977. The effect of cerulenin on sterol biosynthesis in *Saccharomyces cerevisiae*. Lipids **12**:729–731.
27. **Jensen, S. L.** 1964. Carotenoids of photosynthetic bacteria—distribution, structure and biosynthesis, p. 19–33. *In* H. Gest, A. San Pietro, and L. P. Vernon (ed.), Bacterial photosynthesis. Antioch Press, Yellow Springs, Ohio.
28. **Klein, N. C., and L. Mindich.** 1976. Isolation and characterization of a glycerol auxotroph of *Rhodopseudomonas capsulata*: effect of lipid synthesis on the synthesis of photosynthetic pigments. J. Bacteriol. **128**: 337–346.
29. **Laemmli, U. K., and M. Favre.** 1973. Maturation of the head of bacteriophage T4. J. Mol. Biol. **80**:575–599.
30. **Lascelles, J., and J. F. Szilagyi.** 1965. Phospholipid synthesis by *Rhodopseudomonas sphaeroides* in relation to the formation of photosynthetic pigments. J. Gen. Microbiol. **38**:55–64.
31. **LePage, G. A.** 1951. Methods for the analysis of phosphorylated intermediates, p. 190. *In* W. W. Umbreit, R. H. Burris, and J. F. Stauffer (ed.), Manometric techniques and tissue metabolism. Burgess Publishing Co., Minneapolis, Minn.
32. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
33. **Lueking, D. R., R. T. Fraley, and S. Kaplan.** 1978. Intracytoplasmic membrane synthesis in synchronous cell populations of *Rhodopseudomonas sphaeroides*. Fate of "old" and "new" membrane. J. Biol. Chem. **253**: 451–457.
34. **McIntyre, T. M., and R. M. Bell.** 1975. Mutants of *Escherichia coli* defective in membrane phospholipid synthesis. Effect of cessation of net phospholipid synthesis on cytoplasmic and outer membranes. J. Biol. Chem. **250**:9053–9059.
35. **Mindich, L.** 1970. Membrane synthesis in *Bacillus subtilis*. I. Isolation and properties of strains bearing mutations in glycerol metabolism. J. Mol. Biol. **49**:415–432.
36. **Mindich, L.** 1970. Membrane synthesis in *Bacillus subtilis*. II. Integration of membrane proteins in the absence of lipid synthesis. J. Mol. Biol. **49**:433–439.
37. **Mindich, L.** 1975. Studies on bacterial membrane biogenesis using glycerol auxotrophs, p. 429–454. *In* A. Tzagoloff (ed.), Membrane biogenesis: mitochondria, chloroplasts and bacteria. Plenum Publishing Corp., New York.
38. **Niederman, R. A., and K. D. Gibson.** 1971. The separation of chromatophores from the cell envelope in *Rhodopseudomonas sphaeroides*. Prep. Biochem. **1**: 141–150.
39. **Niederman, R. A., and K. D. Gibson.** 1978. Isolation and physicochemical properties of membranes from purple photosynthetic bacteria, p. 79–118. *In* R. K. Clayton and W. R. Sistrom (ed.), The photosynthetic bacteria. Plenum Publishing Corp., New York.
40. **Niederman, R. A., D. E. Mallon, and J. J. Langan.** 1976. Membranes of *Rhodopseudomonas sphaeroides*. IV. Assembly of chromatophores in low aeration cell suspensions. Biochim. Biophys. Acta **440**:429–447.
41. **Nomura, S., T. Horiuchi, T. Hata, and S. Ōmura.** 1972. Inhibition of sterol and fatty acid biosynthesis by cerulenin in cell free systems of yeast. J. Antibiot. **25**:365–368.
42. **Oelze, J., and G. Drews.** 1972. Membranes of photosynthetic bacteria. Biochim. Biophys. Acta **265**:209–239.
43. **Ohno, T., J. Awaya, T. Kesado, and S. Ōmura.** 1974. Target of inhibition by the anti-lipogenic antibiotic cerulenin of sterol synthesis in yeast. Biochem. Biophys. Res. Commun. **57**:1119–1124.

44. **Ōmura, S.** 1976. The antibiotic cerulenin, a novel tool for biochemistry as an inhibitor of fatty acid synthesis. Bacteriol. Rev. **40**:681–697.
45. **Peters, G. A., and R. A. Cellarius.** 1972. Photosynthetic membrane development in *Rhodopseudomonas sphaeroides*. J. Bioenerg. **3**:345–359.
46. **Randall, L. L.** 1975. Insertion of a minor protein into the outer membrane of *Escherichia coli* during inhibition of lipid synthesis. J. Bacteriol. **122**:347–351.
47. **Ray, P. H., T. T. Lillich, and D. C. White.** 1972. Consequences of glycerol deprivation on the synthesis of membrane components in a glycerol auxotroph of *Staphylococcus aureus*. J. Bacteriol. **112**:413–420.
48. **Rouslin, W.** 1977. Some preliminary observations on the formation of mitochondrial and cytoplasmic precursors of electron transfer complexes III and IV in adapting, lipid-limited *Saccharomyces cerevisiae*, p. 511–521. *In* W. Bandlow, R. J. Schweyen, K. Wolf, and F. Kaudewitz (ed.), Mitochondria 1977, genetics and biogenesis of mitochondria. Walter De Gruyter Inc., Elmsford, N.Y.
49. **Schumacher, A., and G. Drews.** 1978. The formation of bacteriochlorophyll-protein complexes of the photosynthetic apparatus of *Rhodopseudomonas capsulata* during early stages of development. Biochim. Biophys. Acta **501**:183–194.
50. **Sistrom, W. R., and R. K. Clayton.** 1964. Studies on a mutant of *Rhodopseudomonas sphaeroides* unable to grow photosynthetically. Biochim. Biophys. Acta **88**: 61–73.
51. **Takamiya, K., and A. Takamiya.** 1969. Light-induced reactions of ubiquinone in photosynthetic bacterium, *Chromatium D.* III. Oxidation-reduction rate in intact cells of *Chromatium D.* Plant Cell Physiol. **10**:363–373.
52. **Takemoto, J., and J. Lascelles.** 1973. Coupling between bacteriochlorophyll and membrane protein synthesis in *Rhodopseudomonas sphaeroides*. Proc. Natl. Acad. Sci. U.S.A. **70**:799–803.
53. **White, D. A., F. R. Albright, W. J. Lennarz, and C. A. Schnaitman.** 1971. Distribution of phospholipid-synthesizing enzymes in the wall and membrane subfractions of the envelope of *Escherichia coli*. Biochim. Biophys. Acta **249**:636–642.
54. **Winrow, M. J., and H. Rudney.** 1971. The biosynthesis of ubiquinone. Methods Enzymol. **18C**:214–232.