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# Control of Bacteriochlorophyll Accumulation by Light in *Rhodobacter capsulatus*

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**The accumulation of bacteriochlorophyll in *Rhodobacter capsulatus* grown either anaerobically or under low aeration is repressed by bright light. It has been proposed that an intact photosynthetic membrane system is required for light-mediated regulation. This was tested by measuring bacteriochlorophyll accumulation in various mutant strains grown under 3% oxygen. Mutants lacking either the reaction center and B875 complexes or the B800-850 complex exhibited normal regulation of bacteriochlorophyll accumulation by light, suggesting that neither photosynthesis nor the photosynthetic membrane system is involved in light-mediated regulation. Bright light did not reduce transcription from the *bchA*, *bchC*, *bchE*, or *bchF* gene. Neither a *bch*$^+$ strain nor *bchG* or *bchH* mutants accumulated greater than normal amounts of any bacteriochlorophyll precursor when grown in bright light, indicating that carbon flow over the bacteriochlorophyll biosynthetic pathway was not being regulated by light intensity. When exposed to bright light, *R. capsulatus* converted aminolevulinate into a colorless compound with $R_f$ values very similar to those of bacteriochlorophyll. These results suggest that in strains grown under low aeration, light intensity controls bacteriochlorophyll accumulation, but does not control bacteriochlorophyll synthesis.**

The regulation of bacteriochlorophyll and carotenoid synthesis in purple non-sulfur photosynthetic bacteria has been the subject of intense investigation for over a quarter century. Cohen-Bazire et al. (6), studying *Rhodobacter sphaeroides* and *Rhodospirillum rubrum*, demonstrated that oxygen and light prevented the accumulation of both bacteriochlorophyll and carotenoids. They hypothesized that the two photopigments were governed by the oxidation state of some component of the electron transport chain. This was based on the assumption that the same mechanism governed regulation by both oxygen and light. Much more recently, Arnheim and Oelze (1) measured intracellular bacteriochlorophyll levels in chemostat cultures with carefully controlled oxygen and light levels. They found that light acted independently of oxygen in the regulation of bacteriochlorophyll accumulation. Further investigation revealed that while both oxygen and light control bacteriochlorophyll accumulation, aminolevulinate synthase responds only to changes in oxygen tension, not to changes in light intensity (11).

Two studies have focused on the control of bacteriochlorophyll formation by oxygen in *Rhodobacter capsulatus*. Clark et al. (4) followed the accumulation of *bch* gene mRNA after a shift from high to low oxygen tension. They found that transcription of the *bch* genes increased when the culture was shifted to 2% oxygen. Using *bch-lacZ* fusions, Biel and Marrs (2) found that transcription of the *bch* genes increased two to fourfold when the oxygen tension was lowered from 23 to 3%. However, they did not detect a change in *bch* gene transcription when the light intensity was changed. It was suggested that the lack of response to light was due to the inability of the fusion strains to synthesize bacteriochlorophyll and thus the pigment-protein complexes.

This study has focused on the control of bacteriochlorophyll accumulation by light in *R. capsulatus* growing under low aeration. Exposure to bright light did not reduce transcription from the *bch* genes, nor did it reduce carbon flow to bacteriochlorophyllide a. These results suggest that in cultures grown under low oxygen, bright light inhibits bacteriochlorophyll accumulation, but does not reduce bacteriochlorophyll synthesis.

## MATERIALS AND METHODS

**Bacterial strains.** The bacterial strains used in this work are described in Table 1. Strain AJB516 was isolated by introducing the conjugative plasmid pRPS404 (10) into strain Y142 and purifying a *crtD puf* recombinant. Strain AJB522 is a spontaneous *bchA*$^+$ revertant of strain BY1651.

**Media and growth conditions.** *R. capsulatus* strains used to measure bacteriochlorophyll accumulation were grown overnight in a malate-minimal salts (RCV) medium (18) and subcultured into RCV medium supplemented with 0.6% glucose, 0.5% pyruvate, and 0.5 M dimethyl sulfoxide (RCV$^+$) to yield an initial density of 25 Klett units (red filter). The culture tubes and sparging apparatus have been described previously (2). The cultures were sparged with a mixture of 92% nitrogen–5% carbon dioxide–3% oxygen. Oxygen tension was measured polarimetrically in the liquid phase of each culture with a Clark-type oxygen electrode (YSI model 53) and remained between 2 and 3%. The cultures were incubated in a glass aquarium at a temperature of 37°C. Light was provided by a bank of three 100-W standard bulbs at a distance of 5 cm. This resulted in a light intensity of 850 W/m$^2$, which has been designated "bright light" throughout this paper. Cultures to be grown in the dark were wrapped in aluminum foil.

**Pigment estimations.** Bacteriochlorophyll concentration was determined by harvesting culture samples (usually 1 ml) and extracting the pellet with an equal volume of acetone-methanol (7:2) as described by Cohen-Bazire et al. (6). Greater than 92% of the bacteriochlorophyll was extracted by this method. Bacteriochlorophyll concentration was estimated at 775 nm with a millimolar extinction coefficient of 76 (5). Protoporphyrin concentration was determined by comparison of the fluorescence of the acetone-methanol extract with protoporphyrin IX standards (Porphyrin Products) in a Perkin-Elmer LS-3 fluorescence spectrophotometer with the

TABLE 1. *R. capsulatus* strains[a]

| Strain | Relevant genotype | Reference |
|---|---|---|
| AJB463 | Φ(*bchH'-lacZ*$^+$)*700 crtD233 hsd-1 str-2* | 2 |
| AJB500 | Φ(*bchG'-lacZ*$^+$)*711 crtD233 hsd-1 str-2* | 2 |
| AJB514 | *crtD233 hsd-1 str-2* | 3 |
| AJB516 | *puf-142 crtD233 str-2* | This study |
| AJB522 | *crtB4 str* | This study |
| BY1651 | *bchA165 crtB4 str* | 10 |
| MW4422 | *crtB4 crtG121 crt-742 puc* | 13 |
| Y142 | *puf-142 str-2* | 8 |

[a] *bchA*, *bchG*, and *bchH* strains are blocked in bacteriochlorophyll biosynthesis and accumulate chlorophyllide a and 2-devinyl, 2-hydroxyethyl chlorophyllide a, bacteriochlorophyllide a, and magnesium protoporphyrin, respectively. *crtB* mutants produce no $C_{40}$ carotenoids. *crtD* and *crtG* strains are blocked in carotenoid biosynthesis and accumulate neurosporene, hydroxyneurosporene, and methoxyneurosporene. *hsd* mutants have enhanced ability to act as recipients in conjugal matings with *Escherichia coli*. *puc* mutants lack the B800-850 complex. *puf* mutants lack the B875 complex and reaction center L and M polypeptides. *str* mutants are streptomycin resistant.

excitation wavelength set at 402 nm and the emission wavelength at 635 nm. Protein concentrations were determined by suspending the pellet in 0.2 N sodium hydroxide and measuring the protein content by the method of Lowry et al. (9), with crystallized bovine serum albumin (Pentex) as the standard. The amount of radioactivity in a band on a thin-layer chromatogram (TLC) was determined by scraping the silica gel into a microcentrifuge tube and extracting twice with methanol. The extracts were spotted on glass fiber filters and counted in a Beckman LS6800 scintillation counter.

**mRNA isolation and RNA-DNA hybridization.** [$^3$H]uridine (20 μCi) was added to cultures grown to 100 Klett units in RCV$^+$. The cultures were incubated for 5 min, harvested, and frozen at −80°C. The pellets were suspended in 1 ml of 50 mM glucose–10 mM EDTA–25 mM Tris chloride (pH 8.0)–2 mg of lysozyme per ml and incubated for 5 min at 4°C. Sodium dodecyl sulfate was added to a final concentration of 1% and the solution was extracted two to three times with an equal volume of water-saturated phenol. The aqueous phase was extracted with chloroform-isoamyl alcohol (24:1), and the nucleic acids were precipitated with an equal volume of isopropanol. The pellet was suspended in 50 mM Tris chloride (pH 7.5)–1 mM EDTA–10 mM magnesium chloride–30 μg of DNase (Boehringer-Mannheim, grade I) per ml and incubated for 30 min at 37°C. The solution was extracted once with phenol and once with chloroform-isoamyl alcohol and precipitated with ethanol. The pellet was suspended in 50 μl of water, and a portion was counted. The volumes of the samples were adjusted so that they contained equal numbers of counts per milliliter. Since the cultures had been pulse-labeled with [$^3$H]uridine, it was possible to dilute the RNA solutions so that they each contained the same concentration of mRNA. Portions of the RNA solution were denatured with glyoxal (17) and pipetted onto a Zeta-Probe membrane with a Bio-Dot Microfiltration apparatus (Bio-Rad Laboratories). The membrane was then baked for 2 h at 80°C. The RNA was hybridized with the nick-translated DNA by the protocol specified by Bio-Rad Laboratories. After hybridization, the membrane was washed twice with 0.3 M sodium chloride–0.03 M sodium citrate–0.1% sodium dodecyl sulfate at room temperature and once in the same buffer at 55°C. Plasmid DNA was isolated by the procedure of Sidikaro and Nomura (14) and nick-translated with [$^{32}$P]ATP with the Bethesda Research Laboratories nick translation reagent kit.

## RESULTS

**Regulation of bacteriochlorophyll accumulation by light in a *bch*$^+$ strain of *R. capsulatus*.** Two cultures of strain AJB514 were grown for one generation in bright light. At that point, one of the cultures was shifted to darkness, and growth of both cultures was continued for another generation. Samples were taken from both cultures throughout this period, and the intracellular bacteriochlorophyll levels were determined. As depicted in Fig. 1, neither culture accumulated bacteriochlorophyll when exposed to bright light. However, the culture shifted to darkness immediately began to accumulate bacteriochlorophyll, eventually accumulating 10-fold more bacteriochlorophyll than the culture exposed to bright light. Absorption and fluorescence spectroscopy failed to detect the accumulation of greater than normal amounts of any bacteriochlorophyll precursor in the extract of the light-grown culture, although a small amount of magnesium porphyrin was detectable by fluorescence spectroscopy in extracts of both dark- and light-grown cultures (excitation maximum, 413 nm; emission maximum, 594 nm).

**Regulation in mutant strains.** Based on the premise that an intact photosynthetic membrane system is required to regulate bacteriochlorophyll synthesis by light, this study was begun by measuring bacteriochlorophyll accumulation in strains deficient in various parts of the photosynthetic apparatus. One feature of *bch* mutants is that they lack pigment-protein complexes (7, 19). Strain AJB516, which lacks the reaction center and B875 complexes due to a mutation in the *puf* operon, regulated bacteriochlorophyll accumulation normally in response to a change in light intensity (Fig. 2). Strain MW4422, which lacks the B800-850 complex, also regulated bacteriochlorophyll accumulation normally, indicating that the three pigment-protein complexes are not directly involved in the regulation of bacteriochlorophyll accumulation by light.

Mutants blocked in bacteriochlorophyll biosynthesis also do not accumulate normal amounts of carotenoids (3). The possibility that the intracellular carotenoid content is impor-

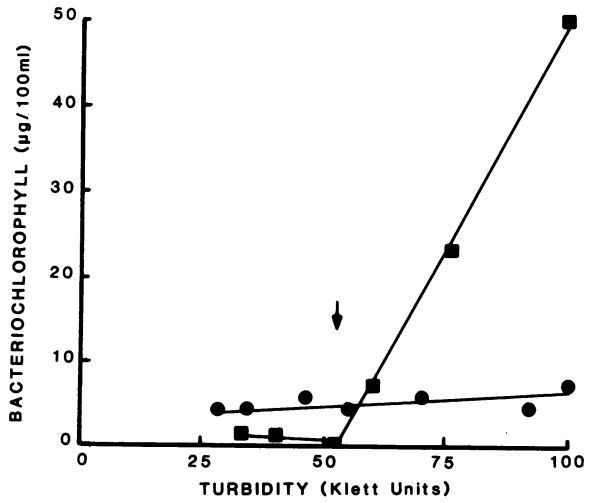


FIG. 1. Bacteriochlorophyll accumulation in the *bch*$^+$ strain AJB514. The strain was grown in bright light and shifted to darkness at the point indicated by the arrow (■) or continuously exposed to bright light (●).

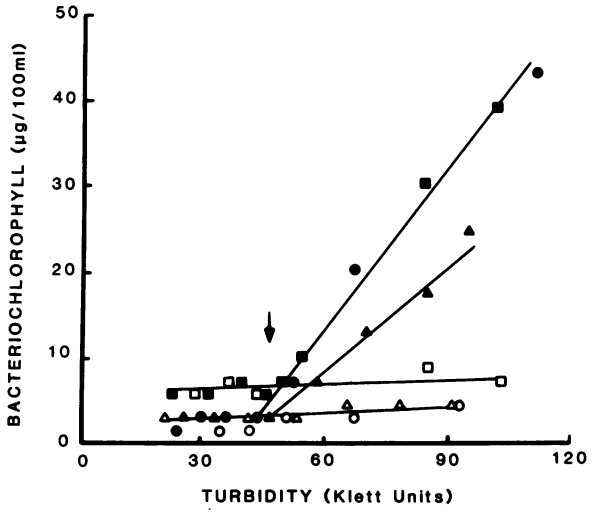


FIG. 2. Bacteriochlorophyll accumulation in mutant strains. Cultures were grown either in bright light for two generations (open symbols) or in bright light for one generation and then in darkness for one generation (solid symbols). The arrow indicates the point at which the cultures were switched to darkness. Symbols: ○, ●, AJB516 (*puf*); △, ▲, AJB522 (*crtB*); □, ■, MW4422 (*puc*).

tant to light regulation was tested by following bacteriochlorophyll accumulation in the carotenoidless strain AJB522 (Fig. 2). This strain showed the normal increase in bacteriochlorophyll accumulation after a shift from bright light to darkness, even though no carotenoids were produced.

**Transcriptional regulation of the *bch* genes.** The fact that strains incapable of photosynthesis and lacking the pigment-protein complexes regulated bacteriochlorophyll accumulation normally suggested that transcription of the *bch* genes is not controlled by light. After growing strain AJB514 for two generations at 3% oxygen in either bright light or darkness, the mRNA was pulse labeled with [$^3$H]uridine and total RNA was isolated as described in Materials and Methods. Three different dilutions of the RNA solutions were spotted on a Zeta-Probe membrane and hybridized with nick-translated pRPSE30 DNA. This plasmid contains both the *bchA* and *bchC* genes (16). After hybridization, the membrane was cut into sections, and the amount of $^{32}$P was determined by Cerenkov counting. As a control, mRNA was isolated from a culture grown in darkness with an initial oxygen tension of 23%. The culture grown in the dark at 23% oxygen had 356 ± 3 cpm, while the culture grown in the dark with 3% oxygen was 1.8-fold higher (641 ± 16 cpm). The other RNA dilutions showed similar differences between the cultures grown in high and low oxygen. This result is in agreement with previous results (2, 4). At all three dilutions, the culture grown under low oxygen in the light had the same amount of hybridization (625 ± 44 cpm) as the culture grown with low oxygen in the dark (641 ± 16 cpm). The experiment was repeated with plasmid pRPSB5, which carries *bchF*, and plasmid pRPSB9, which carries *bchE* (16), as probes. Instead of counting the membrane sections, the membrane was autoradiographed and scanned on a Bio-Rad model 620 densitometer. The culture grown under low oxygen had 2.1-fold more *bchF* mRNA and 1.5-fold more *bchE* mRNA than the culture grown under high oxygen. The culture grown in bright light contained the same amount of *bchE* and *bchF* mRNA as the culture grown in the dark.

**Regulation in *bch* mutants.** The regulation of carbon flow over the bacteriochlorophyll biosynthetic pathway was tested with *bch* mutants. Strain AJB463, a *bchH* mutant that accumulates protoporphyrin IX, and strain AJB500, a *bchG* mutant that accumulates bacteriochlorophyllide a, were grown as described above. As shown in Table 2, exposure to bright light did not reduce the accumulation of either precursor, while the *bch*$^+$ strain accumulated nine times less bacteriochlorophyll when grown in the light than when grown in the dark.

**Conversion of aminolevulinate to bacteriochlorophyll.** To determine whether exposure to bright light was preventing bacteriochlorophyll synthesis, the formation of bacteriochlorophyll from aminolevulinate by dark- and light-grown cultures was investigated. An overnight culture of strain AJB514 in RCV medium was diluted 10-fold into RCV$^+$ medium and split into four cultures. Two cultures were grown in the dark, while the other two were exposed to bright light. When the cultures reached 25 Klett units, 2.5 µCi of [$^{14}$C]aminolevulinate (Amersham; 53 mCi / mmol) was added to one dark-grown and one light-grown culture. Samples were taken from each of the four cultures every half hour, harvested, and extracted with methanol. The extracts from the cultures labeled with [$^{14}$C]aminolevulinate were spotted on a Whatman LK6D silica gel TLC plate and developed with 70% ethanol. Autoradiography revealed that both the dark- and light-grown cultures accumulated one major compound, which comigrated with bacteriochlorophyll ($R_f$ = 0.85). The density of this band increased with time in both extracts (Fig. 3A). In samples from the dark-grown culture, this band was green, whereas no band could be seen in samples from the light-grown culture. A minor band with an $R_f$ intermediate between that of aminolevulinate ($R_f$ = 0.34) and bacteriochlorophyll was observed in all samples. The intensity of this band did not change over time, suggesting that it may represent a bacteriochlorophyll precursor with a large pool size. This compound was not identified. No band corresponding to aminolevulinate was observed in samples from either culture. The bacteriochlorophyll concentration in the samples from the two unlabeled cultures was determined by measuring the absorbance at 775 nm. The intracellular bacteriochlorophyll level increased with time in the culture grown in the dark, while the culture exposed to bright light did not accumulate bacteriochlorophyll (Fig. 3B). Therefore, the radioactive compound that accumulated in the light-grown culture cannot be bacteriochlorophyll, but must be similar to it. Neither petroleum ether-benzene-methanol (4:1:1) nor benzene-ethyl acetate-ethanol (4:1:1) could differentiate between the colorless compound which accumulated in the light-grown cultures and the bacteriochlorophyll which accumulated in the

TABLE 2. Bacteriochlorophyll and precursor levels in strains grown in darkness and bright light

| Strain | Relevant genotype | Pigment concn (nmol/mg of protein) | | |
|---|---|---|---|---|
| | | Bright light | Dark | Ratio, dark/light |
| AJB514 | *bch*$^+$ | 1.5 | 12.9 | 8.6 |
| AJB463 | *bchH* | 0.08 | 0.12 | 1.4 |
| AJB500 | *bchG* | — | — | 0.8[a] |

[a] Due to the lack of a molar absorptivity value for bacteriochlorophyllide a, this value is a ratio of the change in absorbance at 750 nm per milligram of protein under the two growth conditions.

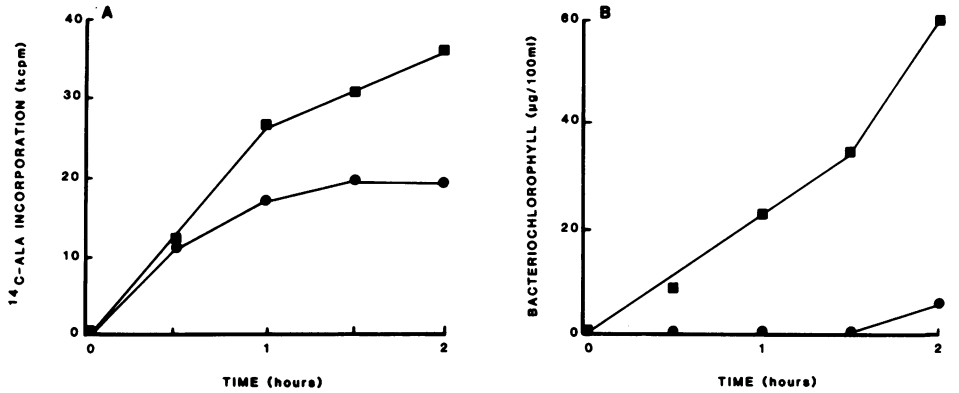


FIG. 3. Conversion of aminolevulinate to bacteriochlorophyll. (A) AJB514 was grown in the dark (■) or in the light (●) in the presence of [$^{14}$C]aminolevulinate. The amount of radioactivity incorporated into the band with an $R_f$ of 0.85 was determined. (B) Amount of bacteriochlorophyll that accumulated in cultures grown in the dark (■) and in the light (●), as determined by absorbance at 775 nm.

dark-grown cultures, indicating that the two compounds are very similar.

Since bacteriochlorophyll is known to be photosensitive, it was of interest to determine whether exposure of bacteriochlorophyll to bright light in vitro might result in the formation of the same compound that accumulated when a culture was exposed to bright light. A concentrated methanolic extract of strain AJB514 was exposed to bright light for several minutes. Portions of the exposed and unexposed extracts were chromatographed in the three solvent systems described above. No bacteriochlorophyll could be detected in the chromatogram of the extract exposed to bright light. However, treatment of the chromatogram with iodine vapors indicated that the extract exposed to bright light contained a colorless compound that comigrated with bacteriochlorophyll in all three systems.

**Net changes in bacteriochlorophyll levels in cultures exposed to bright light.** The experiments described above demonstrated that bacteriochlorophyll continued to be synthesized even in bright light. The question remained whether the bacteriochlorophyll synthesized while the culture was growing in the dark would be degraded after exposure to bright light. In the previous experiments, the low initial intracellular concentration of bacteriochlorophyll made it difficult to determine whether there was any change in the amount of bacteriochlorophyll in the culture during exposure of bright light. Therefore, strain AJB514 was grown for two generations in the dark, diluted to 25 Klett units, and split into two cultures. One culture was grown in the dark, and the other was exposed to bright light. Samples of each culture were taken every half hour, and the bacteriochlorophyll content was determined. Pregrowing the culture in the dark resulted in the accumulation of 58 μg of bacteriochlorophyll per 100 ml of culture and allowed a more sensitive determination of the bacteriochlorophyll level. After 3 h, the bacteriochlorophyll level in the light-grown culture remained unchanged, indicating that exposure to bright light did not result in a decrease in the total amount of bacteriochlorophyll present in the culture.

## DISCUSSION

Measurement of β-galactosidase production in various *bch-lacZ* fusions in *R. capsulatus* demonstrated that exposure to bright light did not reduce transcription (2). This study was complicated by the fact that these strains had blocks in the bacteriochlorophyll biosynthetic pathway and were therefore unable to accumulate the pigment-protein complexes. The inability of bright light to influence transcription from the *bch* genes was explained by suggesting that a photosynthetic membrane system is necessary for light-mediated regulation.

Based on this assumption, the initial experiments were aimed at discovering which components of the photosynthetic membrane system were required for the cell to regulate bacteriochlorophyll synthesis by light. The finding (Fig. 2) that strains lacking the various components of the photosynthetic membrane system regulated bacteriochlorophyll accumulation normally suggested that the earlier findings (2) were not an artifact and that transcription of the *bch* genes was not controlled by light intensity. This was confirmed by measuring transcription from the *bchA*, *bchC*, *bchE*, and *bchF* genes. Cultures grown under bright light had the same amount of mRNA for these genes as cultures grown in the dark. Control experiments indicated that this procedure was sensitive enough to detect the twofold change in mRNA levels between cultures grown in high and low oxygen levels, although it may not be sensitive enough to detect very small changes in mRNA levels.

If transcription of the *bch* genes was not being prevented, perhaps the observed regulation was due to the inhibition of one or more of the bacteriochlorophyll biosynthetic enzymes. If bright light causes a block in the *bch* pathway, greater than normal amounts of some precursor should accumulate. The inhibition of a biosynthetic step appeared unlikely since the *bch*$^+$ strain AJB514 did not accumulate large amounts of bacteriochlorophyll precursors when grown in bright light. However, this possibility was more rigorously tested by measuring the accumulation of bacteriochlorophyll precursors in *bch* mutants. If light prevents carbon flow over the pathway, then a culture grown in bright light should accumulate less precursor than a culture grown in the dark. Two different mutants were tested, one (AJB463) with a block in the first step of the magnesium branch, and the second (AJB500) with a block in the last step of the pathway. Both strains accumulated just as much precursor when grown in bright light as when grown in the dark (Table 2). These results indicate that normal amounts of bacteriochlorophyllide a are being synthesized in the presence of bright light. These results do not, of course, rule out the possibility that the conversion of bacteriochlorophyllide a to bacteriochlorophyll is light sensitive.

Since both enzyme synthesis and carbon flow appeared normal, this raised the possibility that the inability of *R. capsulatus* to accumulate bacteriochlorophyll when exposed to bright light was due to increased degradation of bacteriochlorophyll. This question was addressed in some measure by following the metabolism of radiolabeled aminolevulinate. In dark-grown cultures, bacteriochlorophyll was formed, whereas in light-grown cultures a colorless compound very similar to bacteriochlorophyll was formed (Fig. 3). Absorption and fluorescence spectroscopy clearly demonstrated that this compound was not a normal bacteriochlorophyll precursor. Proof that bacteriochlorophyll is being degraded will have to await identification of the colorless compound. However, it is intriguing that exposure to bright light in vitro caused the conversion of bacteriochlorophyll to a colorless compound that comigrated with it in the three systems tested. The observation that exposure to bright light did not cause a decrease in the amount of bacteriochlorophyll per milliliter of culture makes it tempting to speculate that bacteriochlorophyll synthesized in the dark, and presumably inserted into the membrane, is sheltered from the effects of light.

Since these experiments were conducted in the presence of oxygen, the conclusions do not necessarily apply to anaerobically growing cultures. Further experimentation is required to determine whether light intensity controls bacteriochlorophyll synthesis in photosynthetically growing cultures. Several studies have indicated that light regulates the production of the reaction center and light-harvesting complexes (12, 15). Investigation of the synthesis of these complexes may be helpful in elucidating the mechanism by which the cell senses changes in light intensity.

## ACKNOWLEDGMENT

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