Plant Physiol. (1972) 50, 747–749

Phytol and Bacteriochlorophyll Synthesis in *Rhodopseudomonas spheroides*[1]

Received for publication June 19, 1972

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ABSTRACT

Phytol has been separated and identified in the unsaponifiable lipid fraction from wild type *Rhodopseudomonas spheroides*, but it was not detected in mutant strains blocked at various stages of bacteriochlorophyll synthesis. Incorporation of ^{14}C-acetate into phytol paralleled bacteriochlorophyll synthesis in suspensions of the wild type incubated anaerobically in the light. The addition of chloramphenicol inhibited both processes. It is concluded that phytol formation is tightly coupled to the synhesis of the pyrrole component of bacteriochlorophyll.

Examination of the ultrastructure of mutant strains of *Rhodopseudomonas spheroides* has suggested that the phytol moiety of bacteriochlorophyll is important for the formation of the intracytoplasmic membrane structures found in the wild type (1). In particular, a mutant strain which accumulates bacteriochlorophyllide and is presumably blocked in the phytolation step of bacteriochlorophyll synthesis, does not have these structures.

The biosynthesis of the phytol and pyrrole portions of bacteriochlorophyll proceed by entirely different pathways (2) raising the question of whether they are regulated independently or whether their formation is integrated. We have considered this problem in the present work by analysis of mutants blocked at stages in the synthesis of the pyrrole component of bacteriochlorophyll, to determine whether they continue to form phytol. Also, phytol and bacteriochlorophyll synthesis have been studied with cell suspensions of the wild type to determine whether formation of both components are inhibited by chloramphenicol.

MATERIALS AND METHODS

Organisms and Growth of Cultures. The strains of *R. spheroides* and the growth conditions were as previously described (1). Strain 8-17R was also used. It was a spontaneous revertant derived from mutant 8-17, which fails at the final phytolation step in bacteriochlorophyll synthesis. Strain 8-17R behaved like the wild type with respect to bacteriochlorophyll synthesis and ability to grow photosynthetically.

Extraction and Identification of Phytol. Phytol was extracted from wild type cells grown with low aeration; the cells contained 2 to 4 μmoles of bacteriochlorophyll equivalent to 0.5 to 1 mg of phytol per g dry weight. The cells from 800 ml of culture (about 1 g dry weight of cells) were stirred with 50 ml of chloroform-methanol (2:1) for 1 to 2 hr at room temperature; the extraction was repeated three to four times until colorless extracts were obtained. After centrifugation, the combined extracts were taken to dryness *in vacuo*, dissolved in 100 ml of methanol-water (9:1) containing 5% (w/v) KOH, and refluxed for 30 min. Saturated NaCl (0.1 volume) was added and the mixture extracted twice with 1:1 benzene-petroleum ether (30–60 C boiling point). The aqueous methanol phase was discarded and the organic phase, containing the unsaponifiable lipids, was washed several times with equal volumes of water. It was taken to dryness *in vacuo* and dissolved in 0.5 ml of petroleum ether-diethyl ether (6% v/v). Samples (10 μl or more) were applied to alumina thin layer plates (Eastman Kodak 6062) which were developed in benzene-petroleum ether (1:1). The plates were activated at 100 C for 30 min and stored over $CaCl_2$ prior to use. Phytol was detected on the plates by spraying with 0.5% (w/v) iodine in petroleum ether. Experimental samples were compared with 10 to 20 μg of authentic phytol run concomitantly. Stock solutions of phytol were in petroleum ether (1 mg/ml). For autoradiography, the chromatograms were taped to Kodak no-screen x-ray film for 30 days; film was developed and fixed by standard procedures.

The material designated as phytol on the chromatograms was more positively identified by gas-liquid chromatography and mass spectroscopy. Areas corresponding to authentic phytol were eluted from the alumina with petroleum ether-diethyl ether and concentrated *in vacuo*. A model F and M 402 gas chromatograph with a flame detector heated to 250 C was used (Hewlett-Packard, Palo Alto, Calif.). Chromatography was on a 6-foot column of 3% OV1 on 100 to 200 Gas-chrom C operated at 210 C with nitrogen as carrier gas. Experimental samples were compared with authentic phytol. As a further check of purity, the trimethyl silyl ether derivatives were also compared by gas-liquid chromatography (4). Samples for analysis by mass spectroscopy were eluted from thin layer chromatograms as described above. The eluates were concentrated *in vacuo*, dissolved in benzene, and injected by microsyringe into a Varian gas chromatography with an SE-30 column operating at 175 C. When material was detected by gas chromatography, samples were introduced by direct feed from the chromatograph into a Bell & Howell 21–491 mass spectrometer, operating at 70 electron volts.

Experiments with Cell Suspensions. Bacteriochlorophyll and phytol synthesis were examined with concentrated suspensions of the wild type under conditions similar to those described previously (3). The cells were harvested after growth under low aeration and resuspended to a density of 1 mg dry weight

[1] This work was supported by grants from the National Science Foundation (GB 14967) and the National Institutes of Health (5 ROI AM 11148).

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per ml in malate-glutamate medium supplemented with 1-^{14}C-sodium acetate (1.7 μc/ml; 3 mM). The suspensions were incubated anaerobically in the light at an intensity of 800 ft-c.

Samples were removed at intervals for spectrophotometric determination of bacteriochlorophyll and for determination of ^{14}C incorporation into phytol. For the latter, cells from 10 ml of suspension were extracted to obtain the unsaponifiable lipids, and the entire fraction subjected to thin layer chromatography as described above. Areas on the alumina plates corresponding to authentic phytol were scraped off and added to scintillation vials. The alumina was allowed to elute overnight with 10 ml of toluene-based scintillation fluid (Spectrofluor, Nuclear Chicago, Des Plaines, Ill.) before liquid scintillation counting.

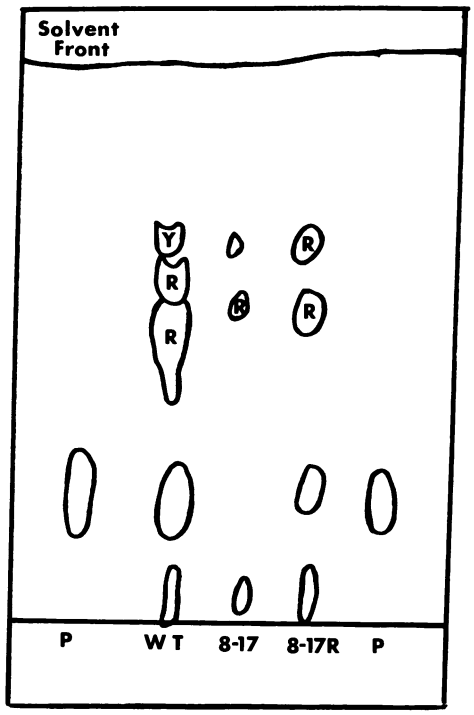

FIG. 1. Thin layer chromatogram of unsaponifiable lipids from the wild type (WT) and strains 8-17 and 8-17R. The cells were grown with low aeration and extracts prepared and chromatographed as described in "Materials and Methods." P: Phytol marker; R and Y denote red and yellow colored spots respectively.

RESULTS AND DISCUSSION

Phytol Detection in Wild Type and Mutant Cells. Phytol was sought in the unsaponifiable lipids of wild type and mutant cells grown under low aeration. Figure 1 depicts the thin layer chromatogram resulting from one of these experiments.

Preparations from the wild type showed a distinct spot corresponding with authentic phytol (R_F 0.22 and 0.23, respectively). It was well separated from the colored spots, presumed to be carotenoids. A similar pattern was found in preparations from revertant strain 8-17R. In contrast preparations from mutants 8-17, 8-32, and 8-29 did not show the phytol spot, though the carotenoid spots were evident. Neither phytol

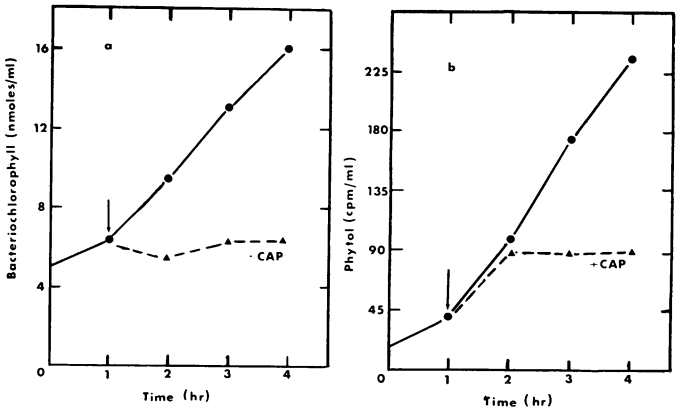

FIG. 3. Effect of chloramphenicol on bacteriochlorophyll and phytol synthesis by suspensions of the wild type. Cell suspensions (1 mg dry wt per ml) were incubated anaerobically in the light in malate-glutamate medium with addition of 1-^{14}C-sodium acetate (1.7 μc per ml; 3 mM). Chloramphenicol (0.2 mM) was added after 1 hr as shown. Samples were withdrawn at intervals and processed for chromatography and determination of radioactivity as described in the "Materials and Methods." ●: Control without chloramphenicol; △: with chloramphenicol (CAP).

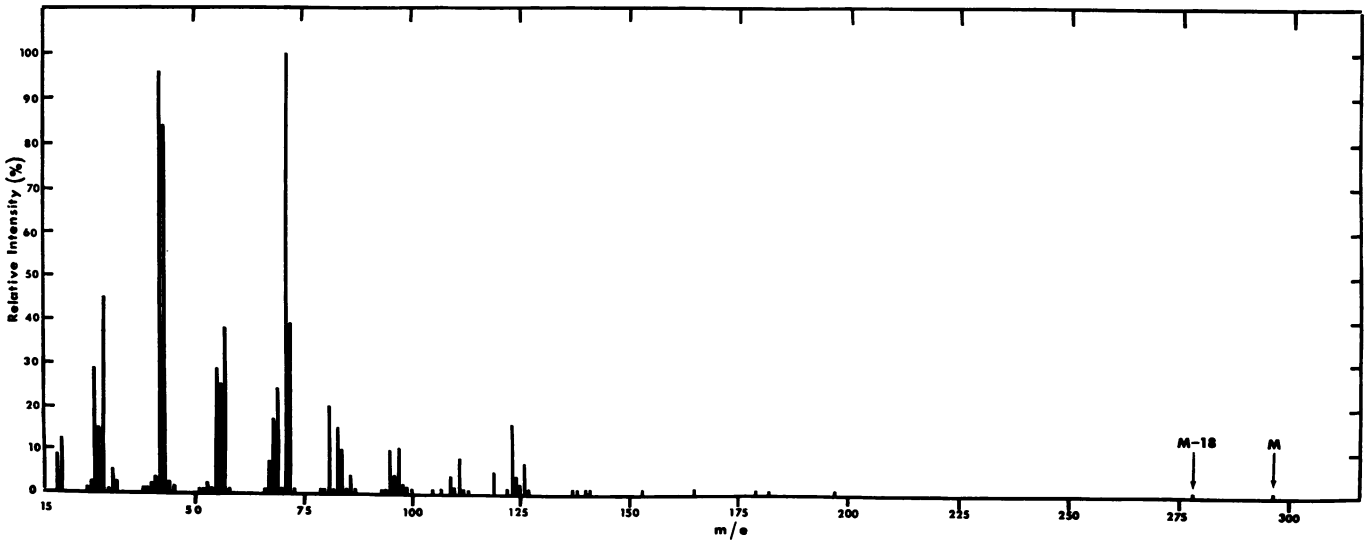

FIG. 2. Mass spectroscopy of phytol preparations from *R. spheroides*. Samples were prepared and analyzed as described in "Materials and Methods."

nor carotenoids were found in preparations from mutant L-57. From recovery experiments with authentic phytol it was calculated that the mutants contained less than 100 μg of phytol per g dry weight of cells; this is less than one-tenth the amount formed by the wild type grown similarly.

The unsaponifiable lipids of the wild type and mutants were also examined by radioautography. In these experiments cultures were grown under low aeration in the malate-glutamate medium, with addition of ^{14}C-acetate when pigmentation became visible. Incubation was continued for 2 hr and the cells were then processed for thin layer chromatography and radioautography. The unsaponifiable lipids from wild type and strain 8-17R showed a radioactive spot corresponding to authentic phytol, whereas preparations from mutants L-57, 8-32, and 8-17 did not. All preparations contained radioactive material which remained at the origin. In addition, the lipid from strain 8-17 showed a radioactive spot with a lower R_F than phytol. Possibly these radioactive materials might be intermediates in triterpenoid synthesis.

Identification of Phytol. The material designated as phytol on chromatography of wild type preparations was more rigorously identified by gas chromatography. It gave a single major peak which corresponded well with that given by authentic phytol (retention times 3 min 23 sec and 3 min 31 sec, respectively). Gas chromatography of the trimethyl silyl ether derivatives gave single major peaks for the experimental and control samples, with retention times of 4 min 20 sec and 4 min 25 sec, respectively. Mass spectroscopic analysis confirmed the identification of phytol (Fig. 2). The molecular ion peak (M) occurs at an m/e value of 296, which is in agreement with the known molecular weight of phytol, 296.52. A second peak occurs at m/e, 278. This peak could arise with the loss of a molecule of water. The peak of greatest relative intensity, m/e, 71, is consistent with several molecular species which could arise during degradation of the parent compound. One possibility is the isoprenoid unit which forms the repeating sequence of the phytol molecules.

Phytol Synthesis by Suspensions of Wild Type. The many observations of inhibition of bacteriochlorophyll formation when protein synthesis is prevented have been based upon spectrophotometric measurement of the pigment. Continuing synthesis of the phytol moiety in the absence of the bacteriochlorophyllide component would not be detected.

The effect of chloramphenicol on both components of bacteriochlorophyll was examined with suspensions of wild type *R. spheroides,* using incorporation of ^{14}C-acetate to follow phytol synthesis (Fig. 3). Synthesis of bacteriochlorophyll (measured spectrophotometrically) and incorporation of ^{14}C-acetate into phytol proceeded at a linear rate when cells were incubated anaerobically in the light. Addition of chloramphenicol abruptly halted pigment synthesis; incorporation of acetate continued for a period, but then ceased completely (Fig. 3).

The failure to find phytol in the mutants suggests that synthesis of the pyrrole and hydrocarbon portions of bacteriochlorophyll are tightly coupled. The inhibitory effects of chloramphenicol on the formation of both components by the wild type suggests that the integrated synthesis is linked closely to protein synthesis. Possibly the formation of a specific membrane protein may be a prerequisite for the continuing synthesis of the pyrrole and phytol moieties.

Acknowledgment—We are indebted to Dr. J. O'Connor for his generous help and advice on lipid methodology.

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