

The Biosynthesis of Bacteriochlorophyll. The Characterization of Latter Stage Intermediates from Mutants of *Rhodospseudomonas spheroides**

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ABSTRACT: Various mutants of the nonsulfur purple bacterium, *Rhodospseudomonas spheroides*, unable to synthesize bacteriochlorophyll have been obtained which excrete tetrapyrrole pigments into the incubation medium. The pigments have been isolated, purified, and characterized chemically as magnesium 2,4-divinylpheoporphyrin a_5 , pheophorbide a , 2-devinyl-2- α -hydroxyethylchlorophyllide a , 2-desacetyl-2- α -hydroxyethylbacteriochlorophyllide, and bacteriochlorophyllide. The products accumulated by the various mutants indicate that the

terminal steps of bacteriochlorophyll biosynthesis proceed in the following sequence: chlorophyllide a \rightarrow 2-devinyl-2- α -hydroxyethylchlorophyllide a \rightarrow 2-desacetyl-2- α -hydroxyethylbacteriochlorophyllide \rightarrow bacteriochlorophyllide \rightarrow bacteriochlorophyll. Compounds likely to be intermediates between magnesium protoporphyrin IX monomethyl ester and magnesium 2,4-divinylpheoporphyrin a_5 and between the latter compound and chlorophyllide a have yet to be found in the photosynthetic bacteria.

It has been amply demonstrated that protoporphyrin is synthesized from δ -aminolevulinic acid through identical intermediates in a variety of organisms, including plants, animals, and microorganisms. The reader is referred to reviews by Bogorad (1966) and Granick (1967). In photosynthetic organisms, the insertion of either iron or magnesium into protoporphyrin determines whether it is to be incorporated into hemoproteins or into chlorophyll.

The postulated pathway of chlorophyll a biosynthesis (Figure 1) is based on studies of compounds accumulated by mutants of *Chlorella* blocked in chlorophyll formation. Compounds which have been positively identified include magnesium protoporphyrin IX, its monomethyl ester, and magnesium 2-vinylpheoporphyrin a_5 (Granick, 1967), and chlorophyllide a (Ellsworth and Aronoff, 1968a). Recently Ellsworth and Aronoff (1968b) have also detected compounds which may be involved in the formation of the cyclopentenone ring. Most of them contained one vinyl and one ethyl group, and hence it was proposed that reduction of the 4-vinyl group to ethyl occurs before formation of the cyclopentenone ring in *Chlorella*.

It seems likely that the biosynthesis of bacterial chlorophylls proceeds through similar, if not identical, intermediates up to the stage of chlorophyllide a . Protoporphyrin IX has been detected in the media of *Rhodospseudomonas spheroides* (Lascelles, 1965) and along with its magnesium derivative, in the green sulfur bacteria (Godnev *et al.*, 1966; Uspenskaya, 1965). Magnesium protoporphyrin IX monomethyl ester has been found in the culture filtrates of *Rhodospseudomonas* species (Cooper, 1963; Jones, 1963a; Lascelles, 1966a) and also in the green photosynthetic bacteria (Richards and Rapoport, 1967).

Magnesium 2,4-divinylpheoporphyrin a_5 , 2-devinyl-2- α -hydroxyethylpheophorbide a , and other uncharacterized pheophorbides were found in cultures of *R. spheroides* grown in the presence of 8-hydroxyquinoline (Jones, 1963b,c, 1964). Various tetrapyrroles are also formed by mutant strains of *R. spheroides* blocked in either bacteriochlorophyll or carotenoid synthesis. Some of these compounds have been separated and characterized as spectroscopically similar to magnesium 2,4-divinylpheoporphyrin a_5 (Stanier and Smith, 1959; Griffiths, 1962; Jones, 1966), pheophorbide a and bacteriopheophorbide (Sistrom *et al.*, 1956), and 2-devinyl-2- α -hydroxyethylpheophorbide a , first reported as chlorobium pheophorbide-650 which it resembles spectroscopically (Griffiths, 1962). The isolation of two mutants of this organism which excrete magnesium 2,4-divinylpheoporphyrin a_5 and 2-devinyl-2- α -hydroxyethylchlorophyllide a into media containing Tween 80 has also been reported (Lascelles, 1966a). It is to be noted that the divinyl derivative of magnesium pheoporphyrin a_5 has been frequently found in *R. spheroides*, suggesting that formation of the cyclopentenone ring precedes reduction of the 4-vinyl group. However, the product of this reduction, magnesium 2-vinylpheoporphyrin a_5 , has not yet been detected in photosynthetic bacteria.

Additional mutants of *R. spheroides* which excrete green pigments have recently been reported (Lascelles and Altshuler, 1967) and the present work describes the identification of some of the accumulated compounds. The pigments include pheophorbide a , 2-desacetyl-2- α -hydroxyethylbacteriochlorophyllide, and bacteriochlorophyllide which may be involved in the latter stages of bacteriochlorophyll biosynthesis (Figure 1). Note that pheophorbide a is shown as its magnesium derivative, chlorophyllide a . The reactions which the various mutants are unable to carry out are indicated in Figure 1.

Experimental Procedures

Cultures and Maintenance. All mutants were derived from *R. spheroides* N.C.I.B. 8253 and were isolated and main-

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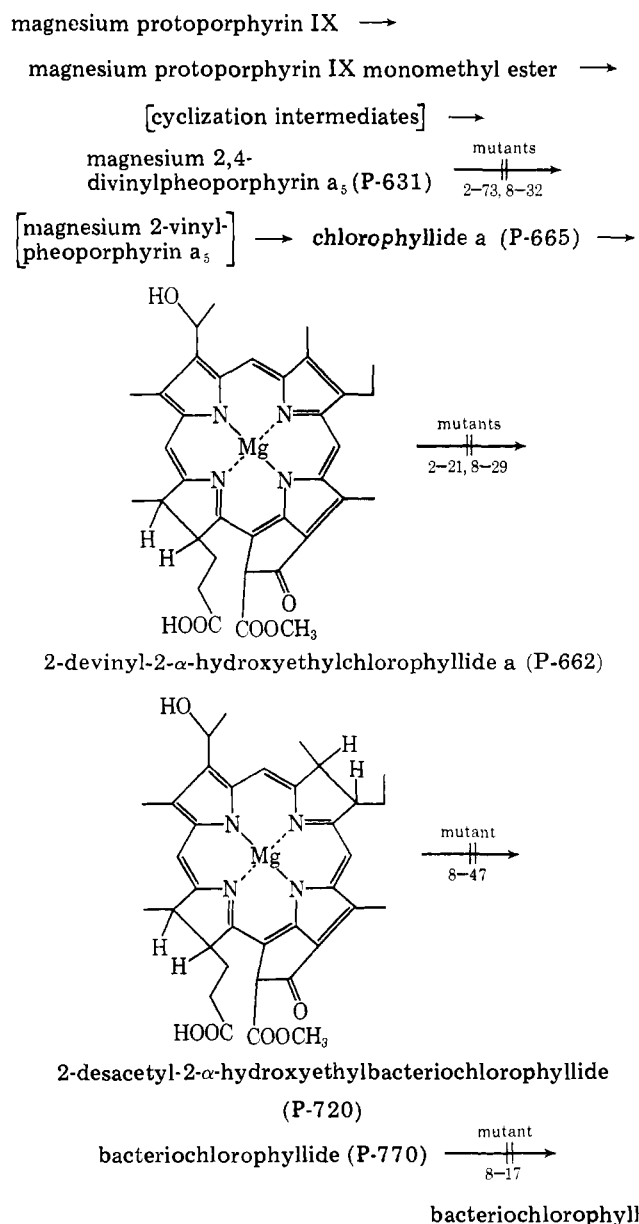


FIGURE 1: The magnesium branch of bacteriochlorophyll biosynthesis.

tained on slope cultures as previously described for mutants 2-21 and 2-73 (Lascelles, 1966a,b). The isolation of subsequent mutants has been reported previously (Lascelles and Altshuler, 1967).

Growth and Incubation. Growth, harvesting, and incubation of the mutants were carried out as previously described for mutants 2-21 and 2-73 (Lascelles, 1966a). The cells were incubated under low aeration for 10–12 hr at 30° in medium A with or without 0.2% (v/v) Tween 80 as indicated. After incubation the cells were removed by centrifugation and the supernatants used for examination of the pigments.

Extraction and Purification of the Pigments. The compounds were first observed in the supernatants after incubation of the various mutants in medium A with Tween 80 and are summarized in Table I. They all contained magnesium as indi-

cated by the spectral shifts upon acid treatment of ethereal extracts of the supernatants (Table I).

Tween 80 interfered with the polyethylene chromatography and purification was therefore carried out with supernatants from cells incubated in medium A without Tween 80. Under these conditions the yields were slightly reduced and the mutants (except for strain 8-32) excreted the magnesium-free pheophorbides. The term "Ph" is used to indicate the magnesium-free derivatives with the numerical designation referring to the red absorption maximum of the corresponding magnesium derivative ("P-") found in supernatants from incubations with Tween 80.

For purification the supernatants were extracted once with ethyl acetate and then brought to pH 5.5 with 85% phosphoric acid. They were then extracted three times with ethyl acetate and the combined extracts were washed with water; acetone and sodium chloride aided in the separation of the phases. The organic phase was evaporated and the residue was used for polyethylene chromatography (Anderson and Calvin, 1962). Aqueous acetone or methanol fractions were extracted into ether with the aid of water, the ether washed with water, dried over anhydrous sodium sulfate, and evaporated. Table II summarizes the pigments found in the various fractions from the mutants.

Preparation of Derivatives. HYDRATION OF VINYL GROUPS. Hydration of vinyl to α -hydroxyethyl groups was accomplished by the method of Falk (1964). First, the HBr addition product was formed and this was subsequently hydrolyzed with 5% aqueous HCl. A few milligrams of sample were dissolved in 1 ml of 50% (w/v) HBr in glacial acetic acid prepared as follows. Anhydrous HBr (0.8 ml) was liquified from HBr gas by cooling with Dry Ice-acetone and then slowly mixed with acetic acid to a volume of 4.4 ml. The sample in HBr-acetic acid was left overnight in the dark at room temperature. The sample was added to 8 ml of 5% aqueous HCl, flushed with argon, and hydrolyzed for 24 hr in the dark at 0°. The product was extracted into ether by dilution of the acid with water, the ether was washed with water, and the spectrum was recorded in ether. The procedure converted pheophorbide a into 2-devinyl-2- α -hydroxyethylpheophorbide a ($\lambda_{\text{max}}^{\text{ether}}$ in $m\mu$ (relative absorption): 658 (1.00), 601 (0.13), 551 (0.06), 531 (0.20), 500 (0.23), 405 (2.15), 370 (1.15), and 310 (0.56)).

DEHYDRATION OF HYDROXYETHYL GROUPS in Vacuo. Thin films of the samples were prepared by rotary evaporation from organic solvents in 25-ml flasks. The flasks were then evacuated to a pressure of between 2 and 3 mm. The flasks were heated for 8 min at $240 \pm 5^\circ$ in a Nujol bath (Fischer *et al.*, 1938), cooled to room temperature, and dry air was admitted. The spectrum of the product was recorded after extraction of the film with ether. This procedure converted the synthetic 2-devinyl-2- α -hydroxyethylpheophorbide a prepared above back into pheophorbide a ($\lambda_{\text{max}}^{\text{ether}}$ in $m\mu$ (relative absorption): 664 (1.00), 609 (0.22), 558 (0.17), 534 (0.29), 503 (0.35), and 409 (2.93)).

CHROMIC ACID OXIDATION OF HYDROXYETHYL GROUPS. The method was employed for conversion of α -hydroxyethyl into acetyl groups and was essentially that employed by Barrett and Clezy (1959). The chromic acid reagent was freshly prepared from 1.4 g of sodium dichromate, 1.2 ml of concentrated sulfuric acid, and 2.9 ml of water. One drop of concentrated sulfuric acid was added to *ca.* 1 mg of the sample in 5 ml of acetone which had been cooled to 0° and flushed with argon,

TABLE I: Pigments Accumulated by Mutants of *R. spheroides* in the Presence of Tween 80.

Mutant	Red Absorption ^a Max (mμ) of Supernatants	Designation of Major Pigment	Major Red Absorption Max (mμ) of Ethereal Extracts ^b		Major Pigment Identified As
			Before Acid	After Acid	
8-32	<i>631</i>	P-631	625	645	Magnesium 2,4-divinylpheoporphyrin <i>a</i> ₃
8-29	<i>662, 617</i>	P-662	654	660	2-Devinyl-2-α-hydroxyethylchlorophyllide <i>a</i>
8-47	<i>720, 669, 620</i>	P-720	715 ^c	712	2-Desacetyl-2-α-hydroxyethylbacteriochlorophyllide
8-17	<i>770, 675, 590</i>	P-770	770	750	Bacteriochlorophyllide

^a Spectra of supernatants after incubation of cells in medium A with Tween 80; the major bands are given in italics. ^b Spectra of ethereal extracts of supernatants before and after acid treatment (Lascelles, 1966a). ^c May have partially lost magnesium.

TABLE II: The Separation of Pigments from Mutants of *R. spheroides* by Powdered Polyethylene Chromatography.

Mutant of <i>R.</i> <i>spheroides</i>	Red Absorption Max (mμ) of Magnesium-Free Tetrapyrroles ^a	Solvents Employed for Elution of Tetrapyrroles during Polyethylene Chromatography ^b				
		% Aqueous Acetone				
		40	50	60	70	
8-32	<i>631</i>	Heme ^c		Ph-770	<i>P-631</i>	
8-29	<i>663, 619</i>	Ph-650 ^d	<i>Ph-662</i>	Ph-665	Other porph ^e	
8-47	<i>710, 674</i>	Ph-650 ^d and Ph-720	<i>Ph-720, Ph-662, and Ph-665</i>			
% Aqueous Methanol						
		50	60	70	80	90-100
8-29	<i>663, 619</i>	Ph-650 ^d		<i>Ph-662</i>	Ph-665	Other porph ^e
8-47	<i>710, 674</i>	Ph-650 ^d	<i>Ph-720</i>	Ph-662	Ph-665 ^f	
8-17	<i>755, 675, ~600</i>			Ph-720	<i>Ph-770</i>	Other porph ^e

^a Spectra of supernatant after incubation in medium A without Tween 80; the major bands are given in italics. ^b The major fraction is in italics. ^c In addition to heme another metalloporphyrin spectroscopically similar to copper protoporphyrin was observed which might possibly be involved in the regulating mechanisms of bacteriochlorophyll biosynthesis (Jones, 1967). ^d A minor component with a spectrum ($\lambda_{\text{max}}^{\text{methanol}}$ in mμ (relative absorption): 650 (1.00), 597 (0.08), 550 (0.03), 497 (0.27), and 393 (3.31)) similar to that reported by Holt (1959) for 9-desoxo-9-hydroxypheophorbide *a*. Kerosene paper chromatography indicated that it contained two hydroxy groups and hence it may have been 2-divinyl-2-α-hydroxyethyl-9-desoxo-9-hydroxypheophorbide *a*. No biological significance has been attributed to this compound. ^e "Other porphyrins" were not determined, but probably consisted of small amounts of Ph-631 and protoporphyrin. ^f In addition, a small amount of a tetrahydroporphyrin with a spectrum similar to 2-desacetyl-2-vinylbacteriopheophorbide was evident running slightly ahead of the Ph-665 band. No biological significance has been attributed to this compound.

followed by one drop of the chromic acid solution. The reaction was complete in about 1 min and the reaction mixture was poured into 20 ml of ether and immediately washed with 0.2% sodium bicarbonate and again with water. The spectrum of the product was recorded in ether or dioxane. The procedure converted hematoporphyrin into 2,4-diacetyldeuteroporphyrin

($\lambda_{\text{max}}^{\text{dioxane}}$ in mμ (relative absorption): 638 (0.24), 584 (0.48), 548 (0.62) 512 (1.00), and 416 (8.79)).

HYDRIODIC ACID-ACETIC ACID PRODUCTION OF PORPHYRIN. The procedure of Fischer and Lakatos (1933) was employed to convert chlorins into porphyrins. Both vinyl and hydroxyethyl groups were reduced to ethyl by this procedure. Phe-

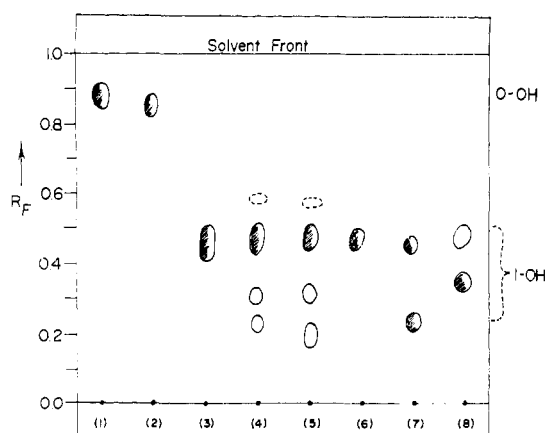


FIGURE 2: Kerosene paper chromatography of chlorin methyl esters. Development for 1 hr with chloroform-kerosene (13:20, v/v) in the presence of chloroform vapor. (1) Pheophorbide a; (2) Ph-665; (3) synthetic 2-devinyl-2- α -hydroxyethylpheophorbide a; (4) the same as part 3, reisolated after shaking 1 day in sterile medium A at 30°; (5) Ph-662 after one polyethylene chromatography; (6) the main fraction of Ph-662 after rechromatography on polyethylene; (7) Ph-665 after hydration with HBr-acetic acid; and (8) Ph-720 after DDBQ oxidation to the chlorin.

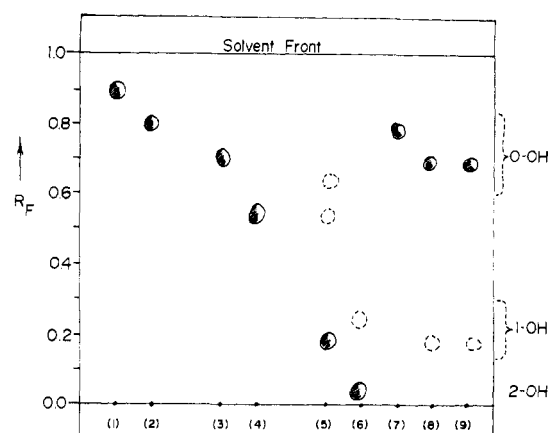


FIGURE 3: Kerosene paper chromatography of porphyrin methyl esters. Development for 1 hr with chloroform-kerosene (13:20, v/v) in the absence of chloroform vapor. (1) Mesoporphyrin; (2) protoporphyrin; (3) pheoporphyrin a_3 ; (4) 2-vinylpheoporphyrin a_3 ; (5) 2-hydroxyethylpheoporphyrin a_3 ; (6) hematoporphyrin (note presence of monohydroxyethylmonovinyldeuteroporphyrin impurity); (7) Ph-631; (8) Ph-662 after oxidation to porphyrin with HI-acetic acid; and (9) Ph-720 after treatment with DDBQ and then oxidation to porphyrin with HI-acetic acid.

ophorbide a (10 mg) was dissolved in 3–4 ml of glacial acetic acid and 10 drops of 47% HI (sp gr 1.5) were added and stirred for 9 min at 60° under argon. The reaction mixture was added to 100 ml of ether and washed three times with 5% K_2HPO_4 and once with water. The spectrum of the product in ether showed the presence of an unidentified chlorin (maximum at *ca.* 650 $m\mu$). The remainder of the spectrum was of the oxorhodo type.¹ The methyl ester of the product was prepared with 5% sulfuric acid in methanol at 0° (Falk, 1964) and was chromatographed on silicic acid-Celite 545 (5:2, w/w; Jeffery, 1963). The main fraction (pheoporphyrin a_3 methyl ester) was eluted with 70–100% chloroform in petroleum ether (bp 60–110°) and the spectrum was recorded ($\lambda_{max}^{chloroform}$ in $m\mu$ (relative absorption): 637 (0.42), 580 (1.10), 563 (1.32), 520 (1.00), and 418 (18.0)).

QUINONE OXIDATION OF TETRAHYDROPORPHYRINS. The procedure was that employed by Lindsay-Smith and Calvin (1966) for oxidation of bacteriochlorophyll to 2-devinyl-2-acetylchlorophyll a. The samples (*ca.* 1 mg) were dissolved in 1 ml acetone to which was added 0.1 ml of solution of 0.01 M DDBQ² in acetone. The reaction was complete in 15 min and the reaction mixture was added to 10 ml ether and washed 10–15 times with 1 ml of water (or until the washings were no longer pink) and the spectrum was recorded in ether. The procedure converted bacteriopheophorbide into 2-devinyl-2-acetylpheophorbide a (λ_{max}^{ether} in $m\mu$ (relative absorption): 682 (1.00), 617 (0.26), 565 (0.11), 540 (0.25), 508 (0.32), 415 (2.68), and 380 (1.88)).

SODIUM BOROHYDRIDE REDUCTION OF CARBONYL FUNCTIONS. The procedure was developed by Holt (1959) for reduction of carbonyl groups in chlorophylls a and b and pheophorbides a

and b. He found that 0.1 mg/ml of sodium borohydride in methanol (with a pyridine catalyst) reduced the 3-formyl group of pheophorbide b to hydroxymethyl, but did not reduce the 9-keto group. However, more concentrated borohydride (1.2 mg/ml in methanol-pyridine) reduced not only the 3-formyl but also the 9-keto groups of both pheophorbides a and b to the corresponding alcohols. In the present work, it was found that a concentration of 0.25 mg/ml of sodium borohydride was required to reduce the 2-acetyl group of bacteriopheophorbide to hydroxyethyl. This concentration was not high enough to reduce the 9-keto group of bacteriopheophorbide; this was accomplished by a concentration of 1.2 mg/ml. Bacteriopheophorbide (1 mg) was dissolved in 3 ml of methanol (plus 9 drops of pyridine) containing 0.25 mg/ml of sodium borohydride. The reaction was complete in 1 min. The reaction mixture was added to ether, washed four times with water, and the spectrum of the product (2-desacetyl-2- α -hydroxyethylbacteriopheophorbide) was recorded (λ_{max}^{ether} in $m\mu$ (relative absorption): 714 (1.00), 659 (0.58), 605 (0.18), 509 (0.81), 479 (0.22), 379 (2.50), and 353 (3.10)). The reaction was repeated with bacteriopheophorbide and 1.2 mg/ml of sodium borohydride in methanol-pyridine for 15 min. The spectrum of the product (2-desacetyl-2- α -hydroxyethyl-9-desoxo-9-hydroxybacteriopheophorbide) was recorded after extraction into ether (λ_{max}^{ether} in $m\mu$ (relative absorption): 716 (1.00), 680 (0.11), 645 (0.14), 615 (0.06), 507 (0.18), 493 (0.23), 465 (0.09), 373 (1.57), and 348 (1.54)).

Paper and Thin-Sheet Chromatography. LUTIDINE PAPER CHROMATOGRAPHY. The ascending chromatographic method of Eriksen (1953) was employed with Whatman No. 1 paper and 2,6-lutidine-water (5:3, v/v) development was for 4 hr in the presence of ammonia vapor to determine the number of unesterified carboxyl groups. In this system, hematoporphyrin had an R_F of 0.76 (2-COOH), pheophorbide a an R_F of 0.90 (1-COOH), and hematoporphyrin dimethyl ester an R_F of 0.99 (O-COOH).

¹ For an explanation of spectral types, see Falk (1964).

² The following abbreviation is used: DDBQ, 2,3-dichloro-5,6-dicyanobenzoquinone.

TABLE III: Spectra of Purified P-631 and Ph-631 from Mutant 8-32.

Compound	Solvent	λ_{\max} (m μ) ^a					
Magnesium 2,4-divinylpheoporphyrin a ₅ ^b	Ether	624 (1.00)	574 (0.58)	537 (0.25)	438 (8.3)		
P-631	Ether	625 (1.00)	573 (0.75)	535 (0.49)	437 (10.0)		
2,4-Divinylpheoporphyrin a ₅ ^b	Ether	642 (0.17)	590 (1.13)	568 (1.68)	527 (1.00)	422 (18.5)	
Ph-631 methyl ester	Chloroform	645 (0.32)	588 (1.09)	568 (1.22)	525 (1.00)	421 (25.0)	

^a Relative absorption in parentheses. ^b Jones (1963b,c).

KEROSENE PAPER CHROMATOGRAPHY. The ascending chromatographic method of Chu *et al.* (1951) as employed by Barrett (1959) was slightly modified for use with the methyl esters of the tetrapyrroles to determine the presence of hydroxy groups. Whatman No. 1 paper was employed with chloroform-kerosene (13:20, v/v) with or without chloroform equilibration; development was for 50–60 min. Samples were esterified by treatment with 5% sulfuric acid in methanol at 0° (Falk, 1964). Acetylation of hydroxy groups was accomplished with a mixture of acetic anhydride-pyridine (1:10, v/v; Barrett, 1959). Sample chromatograms are shown in Figures 2 and 3.

SILICA GEL THIN-SHEET CHROMATOGRAPHY. Eastman silica gel sheets (type K301R2) were employed with acetone-methanol (1:1, v/v). With this system, hematoporphyrin had an R_F of 0.43 (2-COOH), pheophorbide a and bacteriopheophorbide had R_F values of 0.60 and 0.54, respectively (1-COOH), and bacteriopheophytin had an R_F of 0.78 (O-COOH).

Materials. SOLVENTS. All solvents, except kerosene, were reagent grade; the kerosene was a commercial grade. Acetone used as a solvent in the preparation of derivatives was refluxed over potassium permanganate and redistilled. Ether was washed with 10% aqueous ferrous sulfate just before use to remove peroxides.

CHROMATOGRAPHIC ABSORBENTS. Powdered polyethylene (Dohm), silicic acid (J. T. Baker, 100 mesh), and Celite 545 (Johns Manville) were employed for column chromatography.

TETRAPYRROLES. Pheophorbide a was purchased from Fluka AG Chem., Fabrik, Buchs, Switzerland; mesoporphyrin IX from K & K Lab, Inc., Plainview, N. Y., and hematoporphyrin IX and protoporphyrin IX dimethyl ester from Koch-Light Lab Ltd., Colnbrook, England. Bacteriochlorophyll was extracted from *R. spheroides* N.C.I.B. 8253 with methanol (Smith and Benitez, 1955) and purified by polyethylene with 80–90% methanol; bacteriopheophorbide was prepared from it by the method of Fischer and Stern (1940) and was purified by polyethylene chromatography with 80% methanol. The 2-vinylpheoporphyrin a₅ was prepared by the iron-formic acid reduction and air reoxidation procedure of Fischer and Stern (1940), and the product was purified by polyethylene chromatography with acetone; the 2-hydroxyethylpheoporphyrin a₅ was recovered from a 5% aqueous K₂HPO₄ wash of the iron-formic acid reaction mixture employed above which had been left overnight and then extracted with ether.

Instrumental Measurements. Visible spectra were recorded on a Cary Model 14 spectrophotometer with an expanded-scale slide-wire; pH measurements were made with a Beckman Expandomatic pH meter.

Results

Identification of P-631 as Magnesium 2,4-Divinylpheoporphyrin a₅. Mutant 8-32 was incubated in medium A plus Tween 80 and the centrifuged medium was extracted with ether-ethanol (1:1, v/v) by the method of Cooper (1963). The extract contained a residue of Tween 80 and was transferred to ether with the aid of acetone. The spectrum (Table III) corresponded to magnesium 2,4-divinylpheoporphyrin a₅ (Stanier and Smith, 1959; Jones, 1963b,c, 1966). Lutidine paper chromatography of P-631 indicated one free carboxyl group by a spot at R_F 0.88. In an attempt to remove Tween 80 from the sample, the methyl ester of the magnesium-free compound was prepared by 5% sulfuric acid-methanol at 0° was chromatographed on silicic acid-Celite 545 (5:2, w/w; Jeffery, 1963). Impurities were removed from the column with petroleum ether (bp 60–110°) and 50% chloroform in petroleum ether. Ph-631 methyl ester was eluted with chloroform and the spectrum (Table III) was similar to that previously reported for 2,4-divinylpheoporphyrin a₅ (Jones, 1963b,c). The R_F of the methyl ester of Ph-631 was 0.78 with kerosene paper chromatography and was greater than the R_F values of the methyl esters of either pheoporphyrin a₅ or 2-vinylpheoporphyrin a₅ (Figure 3).

Identification of Ph-662 as 2-Devinyl-2- α -hydroxyethylpheophorbide a. Extracts of the incubation medium without Tween 80 of mutant 8-29 were purified by polyethylene chromatography. As indicated in Table II, 50% aqueous acetone or 70% aqueous methanol removed a band with a spectrum similar to the magnesium-free derivative of P-662 previously isolated from mutant 2-21 (Lascelles, 1966a). The sample was rechromatographed on polyethylene and developed with 70% methanol in order to remove traces of impurities (Figure 2; also *cf.* Discussion). Three fractions were eluted: the first corresponded to the R_F 0.31 material, the second to the R_F 0.21 material, and the third to synthetic 2-devinyl-2- α -hydroxyethylpheophorbide a (R_F 0.45) and confirmed the presence of a hydroxy group. It should be noted that the R_F reported by Barrett (1959) for methyl 2-devinyl-2- α -hydroxyethylpheophorbide a (0.10) is much lower than that found in the present work. If chloroform was omitted as an equilibrating solvent, however, all three spots in the unseparated Ph-662 sample appeared as a single elongated spot at R_F 0.09–0.20.

Subsequent chemical work was done with the third (main) fraction (Ph-662). The kerosene paper chromatographic R_F of this fraction was increased to 0.83–0.87 upon acetylation with acetic anhydride-pyridine and upon dehydration *in vacuo*, indicating loss of the free hydroxy group. The spectrum is re-

TABLE IV: Spectra of Purified Ph-662, Ph-665, and Derivatives from Mutant 8-29.

Compound	Solvent	λ_{\max} (m μ) ^a						
2-Deviny-2- α -hydroxyethylpheophorbide a ^b	Ether	659 (1.00)	603 (0.13)	554 (0.07)	532 (0.20)	492 (0.20)	405 (2.22)	
Ph-662	Ether	659 (1.00)	603 (0.19)	552 (0.14)	532 (0.20)	502 (0.22)	405 (2.41)	
Ph-662 after dehydration <i>in vacuo</i>	Ether	667 (1.00)	609 (0.25)	560 (0.21)	536 (0.31)	508 (0.37)	410 (3.25)	
Ph-662 after chromic acid oxidation	Ether	683 (1.00)			540 (0.53)	510 (0.73)	410 (3.86)	375 (2.95)
Ph-662 after HI-acetic acid oxidation	Ether	632 (0.26)	581 (1.22)	560 (1.47)	520 (1.00)		416 (18.4)	
Pheophorbide a ^c	Ether	667 (1.00)	610 (0.15)	560 (0.06)	534 (0.21)	505 (0.23)	408 (2.09)	
Ph-665	Ether	667 (1.00)	609 (0.19)	555 (0.09)	534 (0.21)	507 (0.25)	408 (2.42)	
Ph-665 after hydration with HBr-acetic acid	Ether	659 (1.00)	601 (0.22)	555 (0.08)	528 (0.16)	498 (0.25)	405 (2.33)	375 (sh)
Ph-665 after HI-acetic acid oxidation	Ether	648 (0.40)	635 (0.37)	587 (1.17)	563 (1.35)	525 (1.00)	417 (12.1)	

^a Relative absorption in parentheses. ^b Jones (1964). ^c Smith and Benitez (1955).

corded in Table IV and is identical with the spectrum of 2-deviny-2- α -hydroxyethylpheophorbide a (Jones, 1964).

Lutidine paper chromatography (R_F 0.88) and silica gel thin-sheet chromatography (R_F 0.63) of Ph-662 indicated one free carboxyl group. After dehydration *in vacuo* the spectrum of Ph-662 shifted from 659 m μ to that of pheophorbide a (667 m μ) indicating the dehydration of one hydroxyethyl group (Table IV). A chromic acid oxidation of Ph-662 was carried out and the spectrum of the product is recorded in Table IV. The increase in conjugation (to 683 m μ) indicated that the resulting acetyl group was due to oxidation of one hydroxyethyl group, and that it was in the α position to the tetrapyrrole nucleus. Ph-662 was converted into a porphyrin by treatment with HI in acetic acid. The product was purified by polyethylene chromatography and the main fraction eluted with 90–100% methanol. The spectrum of the product (Table IV) was identical with pheoporphyrin a₃ produced from pheophorbide a by the same procedure (*cf.* Experimental Section). Kerosene paper chromatography of the methyl ester indicated that the hydroxyethyl group had been converted into ethyl by the reaction (Figure 3).

The first two fractions had spectra very similar to Ph-662 except that the Soret peak was shifted to 398 m μ . In all other ways tested (dehydration *in vacuo*, acetylation, chromic acid oxidation, and HI-acetic acid oxidation to porphyrin), the first two fractions behaved similarly to Ph-662 (except that the fraction 1 methyl ester *O*-acetyl derivative had an R_F of 0.67 in kerosene paper chromatography).

Identification of Ph-665 as Pheophorbide a. During chromatography of extracts from mutant 8-29, a minor component which trailed the main (Ph-662) band was eluted with either 60% aqueous acetone or 80% aqueous methanol. Upon rechromatography of this fraction on polyethylene a second component was revealed. The latter, partially separated during rechromatography on polyethylene, had a spectrum similar to the main fraction but with a Soret peak shifted to 402 m μ . Complete separation of this component from the main frac-

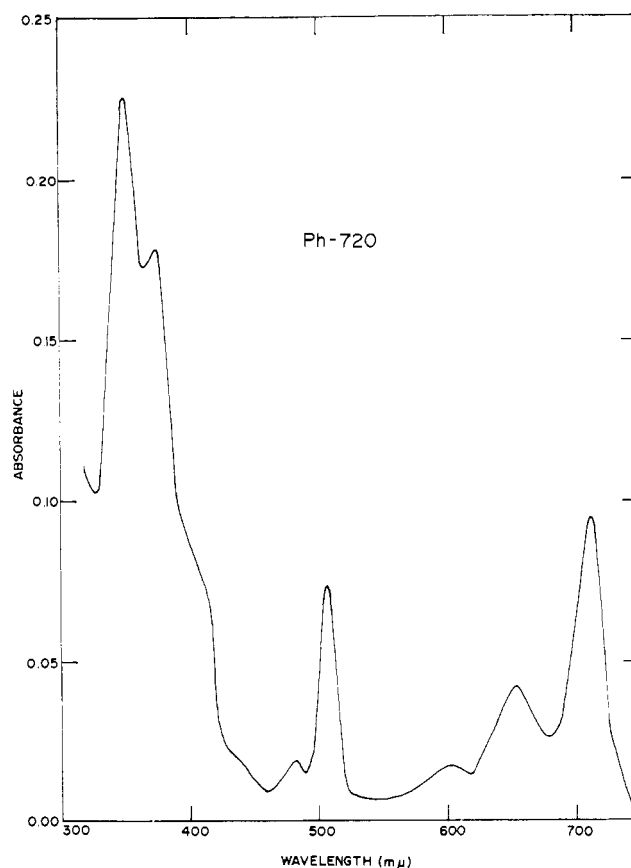
tion was not obtained. The main fraction was named Ph-665 and its spectrum corresponded to that of pheophorbide a (Table IV). Lutidine paper chromatography (R_F 0.87) and silica gel thin-sheet chromatography (R_F 0.60) indicated one free carboxyl group. The kerosene paper chromatographic R_F of the methyl ester was identical with methyl pheophorbide a (Figure 2). Ph-665 was reacted with 50% HBr in acetic acid, followed by hydrolysis in 5% HCl. The product was chromatographed on polyethylene and the main band eluted with 50% acetone. The product had a spectrum corresponding to 2-deviny-2- α -hydroxyethylpheophorbide a (Table IV). Kerosene paper chromatography of the methyl ester revealed the presence of two spots in the monohydroxy region of the chromatogram (Figure 2) corresponding to synthetic 2-deviny-2- α -hydroxyethylpheophorbide a and to the fraction 2 impurity (with R_F 0.21) of Ph-662 (*cf.* Discussion). The R_F values of both spots increased to 0.83–0.85 both upon dehydration *in vacuo* and upon acetylation with acetic anhydride-pyridine.

Reaction of Ph-665 with HI in acetic acid converted it into a porphyrin with an oxorhodo-type spectrum.¹ The sample was purified by polyethylene chromatography. After elution of bands with spectra similar to mesopheophorbide and acetyl-pheoporphyrin (from the oxoreaction of Fischer; *cf.* Seely, 1966) with 70–80% methanol, a fraction with a spectrum similar to pheoporphyrin a₃ was eluted with methanol and acetone (Table IV). The product still contained a small amount of an unidentified chlorin (maximum at 648 m μ) also noticed during the same reaction with pheophorbide a (*cf.* Experimental Section).

Identification of Ph-720 as 2-Desacetyl-2- α -hydroxyethylbacteriopheophorbide. Extracts of the incubation medium without Tween 80 of mutant 8-47 were purified by polyethylene chromatography. As indicated in Table II, the main band was eluted with 40–50% acetone. However, methanol was found to be a much more effective solvent in separating the main component, Ph-720, from the minor components, Ph-650, Ph-662, and Ph-665. The spectrum of the major frac-

TABLE V: Spectra of Purified Ph-720 and Derivatives from Mutant 8-47.

Compound	Solvent	λ_{\max} (m μ) ^a									
Ph-720	Ether	712 (1.00)	655 (0.44)	605 (0.18)	508 (0.78)	477 (0.21)	378 (1.88)	350 (2.37)			
Ph-720	Methanol	708 (1.00)	649 (0.51)	600 (0.23)	511 (0.88)	480 (0.25)		348 (2.96)			
Ph-720 after dehydration <i>in vacuo</i>	Ether	725 (1.00)	664 (0.56)	610 (0.17)	510 (0.72)	480 (0.24)	381 (2.40)	353 (2.87)			
Ph-720 after dehydration <i>in vacuo</i> then DDBQ	Ether		667 (1.00)	608 (0.21)	563 (0.21)	503 (0.41)	408 (3.26)				
Ph-720 after DDBQ	Ether		660 (1.00)	601 (0.11)	529 (0.16)	501 (0.23)	405 (2.19)				
Ph-720 after DDBQ, then dehydration <i>in vacuo</i>	Ether		667 (1.00)	605 (0.35)	560 (0.33)	506 (0.44)	411 (3.94)				
Ph-720 after DDBQ, then chromic acid oxidation	Ether		683 (1.00)	625 (0.10)	566 (0.06)	510 (0.26)	415 (2.71)	380 (1.86)			
Ph-720 after concentrated NaBH ₄	Methanol	718 (1.00)	648 (0.28)	600 (0.16)	511 (0.37)	492 (0.38)	465 (sh)	371 (2.53)	348 (2.79)		
Ph-720 after concentrated NaBH ₄ , then DDBQ	Ether		658 (1.00)	597 (0.38)	528 (0.52)	498 (0.64)	401 (3.45)				

^a Relative absorption in parentheses.FIGURE 4: The spectrum of 2-desacetyl-2- α -hydroxyethylbacteriopheophorbide (Ph-720) from mutant 8-47 in ether.

tion (Figure 4 and Table V) was unknown but indicated that the compound was a tetrahydroporphyrin with less conjugation than bacteriopheophorbide. The following data identified Ph-720 as 2-desacetyl-2- α -hydroxyethylbacteriopheophorbide: lutidine paper chromatography (R_F 0.90) and silica gel thin-sheet chromatography (R_F 0.62) indicated the presence of one free carboxyl group. Dehydration of Ph-720 *in vacuo* yielded a product with a spectrum shifted from 712 to 725 m μ , indicating the formation of one vinyl group. Treatment of the product with DDBQ converted it into a chlorin with a spectrum similar to pheophorbide a (maximum at 667 m μ).

Treatment of Ph-720 itself with DDBQ converted it into a chlorin with a spectrum identical with 2-devinyl-2- α -hydroxyethylpheophorbide a (maximum at 659 m μ). Dehydration *in vacuo* of this chlorin also produced a compound with a spectrum similar to pheophorbide a (maximum at 667 m μ). Chromic acid oxidation of the chlorin produced by DDBQ oxidation of Ph-720 yielded a compound with a spectrum similar to 2-devinyl-2-acetylpheophorbide a (maximum at 682 m μ) and confirmed the presence of only one α -hydroxyethyl group. Further confirmation of the presence of the hydroxy group was provided by kerosene paper chromatography of the methyl ester of the chlorin (Figure 2; also *cf.* Discussion). The R_F was increased by dehydration *in vacuo* to 0.86 and by acetylation with acetic anhydride-pyridine to 0.68. Oxidation of the chlorin with HI in acetic acid produced a porphyrin, and the methyl ester had an R_F of 0.68 with kerosene paper chro-

TABLE VI: Spectra of Purified Ph-770 and Derivatives from Mutant 8-17.

Compound	Solvent	λ_{\max} (m μ) ^a				
Bacteriopheophorbide ^b	Acetone	745 (1.00)	673 (0.23)	605 (0.09)	522 (0.54)	382 (1.10) 357 (2.03)
Ph-770	Acetone	745 (1.00)	673 (0.36)	600 (0.14)	523 (0.54)	383 (1.45) 357 (2.47)
Ph-770	Ether	750 (1.00)	668 (0.39)	617 (0.08)	524 (0.49)	383 (1.60) 357 (2.31)
2-Devinyl-2-acetylphoe-						
phorbide a ^c	Acetone		680 (1.00)	619 (0.19)	542 (0.26)	380 (1.72)
Ph-770 after DDBQ	Ether		682 (1.00)	617 (0.26)	540 (0.25)	380 (1.88)
Ph-770 after dilute NaBH ₄	Ether	710 (1.00)	655 (0.39)	602 (0.14)	509 (0.81)	378 (1.45) 350 (2.38)
Ph-770 after dilute NaBH ₄ , then DDBQ	Ether		659 (1.00)	603 (0.20)	555 (0.16)	370 (sh)
Ph-770 after concentrated NaBH ₄	Methanol	715 (1.00)	645 (0.34)	611 (0.18)	513 (0.41)	369 (2.64) 347 (3.02)
Ph-770 after concentrated NaBH ₄ , then DDBQ	Ether		658 (1.00)	600 (0.19)	550 (0.16)	401 (3.30)

^a Relative absorption in parentheses. ^b Eimhjellen *et al.* (1963). ^c Lindsay-Smith and Calvin (1966).

matography, similar to that of pheoporphyrin a₅ (Figure 3), and confirmed the observation made with Ph-662 that the hydroxyethyl group is converted into ethyl by this procedure.

Reduction of Ph-720 for 15 min with 1.2 mg/ml NaBH₄ in methanol-pyridine produced a compound tentatively identified as 2-desacetyl-2- α -hydroxyethyl-9-desoxo-9-hydroxybacteriopheophorbide. A similar compound was produced from bacteriopheophorbide with 1.2 mg/ml of NaBH₄ (*cf.* Experimental Section). With slightly less concentrated NaBH₄ (0.25 mg/ml in methanol) reaction for 1 min with bacteriopheophorbide (*cf.* Experimental Section) yielded a compound spectroscopically similar to Ph-720, indicating reduction of the 2-acetyl to hydroxyethyl, but no reduction of the 9-keto group. All of the spectra mentioned above are summarized in Table V.

Identification of Ph-770 as Bacteriopheophorbide. Extracts of the incubation medium without Tween 80 of mutant 8-17 were purified by polyethylene chromatography. As indicated in Table II, the main band was eluted with 80% methanol. The spectrum in acetone (Table VI) was almost identical with the spectrum published for bacteriopheophorbide (Eimhjellen *et al.*, 1964). Lutidine paper chromatography (R_F 0.90) and silica gel thin-sheet chromatography (R_F 0.57) indicated the presence of one free carboxyl group. Treatment of Ph-770 with DDBQ yielded a compound spectroscopically identical with 2-devinyl-2-acetylphoeophorbide a (maximum at 682 m μ). Reduction of Ph-770 with dilute NaBH₄ (0.25 mg/ml in methanol) produced a compound with a spectrum similar to 2-desacetyl-2- α -hydroxyethylbacteriopheophorbide (Ph-720). The product was purified by polyethylene chromatography by elution with 60% methanol, and its spectrum is recorded in Table VI. Treatment of this product with DDBQ yielded a chlorin with a spectrum identical with 2-devinyl-2- α -hydroxyethylphoeophorbide a (Ph-662). Kerosene paper chromatography of the methyl ester of this chlorin indicated one hydroxy group with an R_F of 0.35 similar to the chlorin noticed after DDBQ treatment of Ph-720 (Figure 2; also *cf.* Discussion). Reduction of Ph-770 with concentrated NaBH₄ (1.2 mg/ml in methanol-pyridine) produced a compound spectroscopically similar to one produced by similar treatment of Ph-720 (*cf.* above) and tentatively identified as 2-desacetyl-2- α -hydroxyethyl-9-desoxo-9-hydroxybacteriopheophorbide. Treatment of either of these products (produced by reaction of concentrated NaBH₄ with either Ph-720 or Ph-770) with DDBQ yielded chlorins with similar spectra. These spectra were in turn similar to the spectrum of 2-devinyl-2- α -hydroxyethylphoeophorbide a (Ph-662) and indicated that the DDBQ treatment not only oxidized the tetrahydroporphyrin to the chlorin but also reoxidized the 9-hydroxy group to the ketone. Kerosene paper chromatography of the methyl esters of both these chlorins showed predominately the presence of monohydroxy compounds, but the chlorin from Ph-770 showed in addition a spot at R_F 0.11 indicating the presence of two hydroxy groups due to incomplete oxidation of the 9-hydroxy group. All of the spectra mentioned above are summarized in Table VI.

Discussion

The finding that magnesium 2,4-divinylpheoporphyrin a₅ (P-631) is accumulated by mutant 8-32 adds to previous observations of formation of the divinyl derivative by *R. spher-*

oides (Stanier and Smith, 1959; Griffiths, 1962; Jones, 1963b, c, 1966; Lascelles, 1966a), and rigorous confirmation of its structure was therefore not attempted. The frequent occurrence of the divinyl compound strongly suggests that formation of the cyclopentenone ring precedes reduction of the 4-vinyl group during bacteriochlorophyll synthesis in *R. spheroides*. This contrasts with the observations of Ellsworth and Aronoff (1968b) with *Chlorella* mutants, which indicated that reduction of the 4-vinyl group preceded cyclization during chlorophyll a synthesis in that organism. Ricketts (1966) has, however, reported magnesium 2,4-divinylpheoporphyrin a_8 in some unicellular marine flagellates and a protein complex of the corresponding phytol ester has been detected in seed coats of *Cucurbita pepo* (Jones, 1966). Possibly the biosynthesis of chlorophyll a in these plant tissues is similar to that in *R. spheroides* in that cyclization precedes 4-vinyl reduction.

The identification of the major pigment accumulated by mutant 8-29 as 2-devinyl-2- α -hydroxyethylchlorophyllide a (P-662) is supported by previous observations of this compound or its magnesium-free derivative in cultures of *R. spheroides* (Jones, 1963b, 1964; Lascelles, 1966a). Its immediate precursor can be presumed to be chlorophyllide a. This compound was not shown directly but the magnesium-free derivative, pheophorbide a (Ph-665) was identified as a relatively minor product in Tween-free supernatants from mutant 8-29 and it was also found in preparations from mutant 8-47. Compounds similar to pheophorbide a have been reported in cultures of *R. spheroides* but have not been characterized chemically (Sistrom *et al.*, 1956; Jones, 1963b). Direct confirmation of the presence of chlorophyllide a in the mutants was not made because of the difficulty in purifying extracts containing Tween 80. Chlorophyllide a is readily demonstrable in etiolated leaves which have been briefly exposed to light and the pigments quickly isolated (Smith, 1960). It has also been found in a *Chlorella* mutant unable to synthesize chlorophyll a (Ellsworth and Aronoff, 1968a).

Samples of Ph-662 from mutant 8-29, which had been purified once only by polyethylene chromatography, revealed three spots in the monohydroxyl region of kerosene paper chromatograms of the methyl esters (Figure 2). The major one (R_F 0.45) corresponded to synthetic 2-devinyl-2- α -hydroxyethylpheophorbide a formed from pheophorbide a with HBr-acetic acid. The other spots (R_F 0.31 and 0.21) also appeared in a sample of the synthetic material which had been reisolated after shaking for 1 day at 30° in sterile medium A, and probably represented "altered" forms of the chlorin similar to those described by Strain and Svec (1966) for chlorophyll a. One of the artifacts may correspond to an unidentified pheophorbide (fraction 1c) noted by Sistrom *et al.* (1956) with a Soret peak at 400 m μ . Hydration of Ph-665 with HBr-acetic acid also gave a product which revealed two spots on paper chromatography (Figure 2). One was true 2-devinyl-2- α -hydroxyethylpheophorbide a, and the other (R_F 0.21) was probably due to hydration of an altered chlorin. However, it is possible that the latter compound was a 2,4-dihydroxyethylchlorin. Such a compound could have arisen from the HBr-acetic acid hydration of 4-desethyl-4-vinylpheophorbide a, possibly the "second component" noticed during purification of Ph-665 (*cf.* Results). The magnesium derivative of this compound could be the actual biosynthetic intermediate after magnesium 2,4-divinylpheoporphyrin a_8 , which has yet to be determined in photosynthetic bacteria (Figure 1).

The chemical data adequately demonstrate that Ph-720, isolated from mutant 8-47, is 2-desacetyl-2- α -hydroxyethylbacteriopheophorbide. Kerosene paper chromatography of the chlorin produced by reaction of Ph-720 with DDBQ showed that the major product had an R_F of 0.37 (Figure 2). The following data showed that it was not the artifact (R_F 0.31) isolated during purification of Ph-662: (a) the chlorin from Ph-720 had a Soret peak at 405 m μ , and was nearly identical with that of Ph-662 (Table V); and (b) it reacted more completely with chromic acid and produced a purer product (2-devinyl-2-acetylpheophorbide) than did Ph-662 or its "altered" fractions, or synthetic 2-devinyl-2- α -hydroxyethylpheophorbide a (W. R. Richards, unpublished observations). It is therefore probable that the product of the oxidation of Ph-720 with DDBQ was a mixture of the two chlorins which are possible, and that the 3,4-dihydroporphyrin isomer (corresponding to the spot at R_F 0.37) predominated over the 7,8-dihydroporphyrin isomer (R_F 0.45).

The identification of bacteriochlorophyllide (P-770) from mutant 8-17 confirms that phytolization is the last step in the biosynthesis of bacteriochlorophyll. Bacteriopheophorbide has been detected spectroscopically in carotenoidless mutants of *R. spheroides* by Sistrom *et al.* (1956).

The compounds accumulated by the various mutants of *R. spheroides* justify the biosynthetic sequence from magnesium protoporphyrin IX to bacteriochlorophyll outlined in Figure 1 except for possible cyclization intermediates between magnesium protoporphyrin IX monomethyl ester and magnesium 2,4-divinylpheoporphyrin a_8 , and for the intermediate between the latter compound and chlorophyllide a. It should be noted that 2-devinyl-2- α -hydroxyethylchlorophyllide a is likely to be the branch point for the biosynthesis of the chlorobium chlorophylls (Richards and Rapoport, 1967) in the green sulfur bacteria, which are also able to synthesize bacteriochlorophyll. It is not clear why the mutants excreted the magnesium derivatives in the presence of Tween 80, whereas magnesium-free compounds accumulated in its absence. Possibly, the detergent may release the magnesium intermediates from their active sites; alternatively, the magnesium chelates are more unstable in aqueous media without Tween 80.

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