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A Possible Alternate Pathway of Bacteriochlorophyll Biosynthesis in a Mutant of *Rhodopseudomonas sphaeroides*†

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ABSTRACT: A previously uncharacterized bacteriochlorophyll-less mutant (mutant 8) of *Rhodopseudomonas sphaeroides* has been found to excrete a tetrapyrrole–protein complex into the incubation medium. The structure of the major pigment of the complex was characterized as 2-desacetyl-2-vinylbacteriopheophorbide. The corresponding magnesium derivative does not fit into the currently proposed biosynthetic pathway for bacteriochlorophyll, and thus may indicate the existence of an alternate pathway of bacteriochlorophyll synthesis in this organism. Such an alternate pathway would be possible if reduction from the chlorin to the tetrahydroporphyrin stage can occur either before or after hydration of the 2-vinyl substituent of chlorophyllide *a* to an α-hydroxyethyl group.

A plausible sequence for the latter stages (or magnesium branch) of Bchl[1] synthesis is shown in Figure 1. This pathway was proposed on the basis of work carried out with mutants of *Rhodopseudomonas sphaeroides* unable to synthesize either Bchl (Griffiths, 1962; Lascelles, 1966; Lascelles and Altshuler, 1967; Richards and Lascelles, 1969) or carotenoids (Sistrom et al., 1956; Stanier and Smith, 1959), and with the wild-type strain inhibited by 8-hydroxyquinoline (Jones, 1963a,b, 1964, 1967). In all of these studies, tetrapyrrole pigments were excreted into the growth medium. After isolation and identification, the compounds were arranged in a logical sequence which would accomplish the synthesis of Bchl (Figure 1). In all cases, the true intermediates were assumed to be the magnesium chelates, whether they or the corresponding magnesium-free derivatives were actually isolated. Lascelles (1966) observed that the pigments were actually excreted as lipoprotein-bound complexes, and suggested that these might represent a natural "carrier protein" complex required for Bchl synthesis. No intermediate (assumed to be magnesium 2-vinylpheoporphyrin a_5) between P-631 and P-665 or any intermediates involved in the cyclization of the cyclopentenone ring have been identified in *R. sphaeroides* (Figure 1). The addition of phytol was assumed to be the final step in Bchl formation, since none of the intermediates contained a phytyl ester. The only enzyme of the magnesium branch to be demonstrated in cell-free extracts has been magnesium-protoporphyrin:*S*-adenosylmethionine methyltransferase (EC 2.1.1.11) by Gibson et al. (1963). Lascelles and Hatch (1972) have pointed out, however, that the mutants should be good sources of intermediates for future enzymatic studies.

In studies with closely related strains of *Athiorhodaceae*, Krasnovskii et al. (1970) have detected compounds spectroscopically similar to magnesium 2,4-divinylpheoporphyrin a_5, chlorophyll *a*, and 2-devinyl-2-α-hydroxyethylchlorophyll *a* in mutants of *Rhodopseudomonas palustris*. The pigments were found to be mixtures of the phytyl esters and the corresponding nonphytylated derivatives. Drews et al. (1971) have isolated a pigment–protein complex from a mutant of *Rhodopseudomonas capsulata* containing the phytyl esters of both magnesium 2-vinylpheoporphyrin a_5 and the corresponding magnesium-free derivative. Oelze and Drews (1970) have also isolated a pigment–protein complex from a mutant of *Rhodospirillum rubrum*. However, in this case the pigments excreted in the complex were the nonesterified pheophorbide *a* and 2-devinyl-2-α-hydroxyethylpheophorbide *a*. Schick and Drews (1969) had earlier described another mutant of *R. rubrum* which excreted a much different pigment–protein complex containing bacter-

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[1] Abbreviations used are: Bchl, bacteriochlorophyll; DDBQ, 2,3-dichloro-5,6-dicyanobenzoquinone.

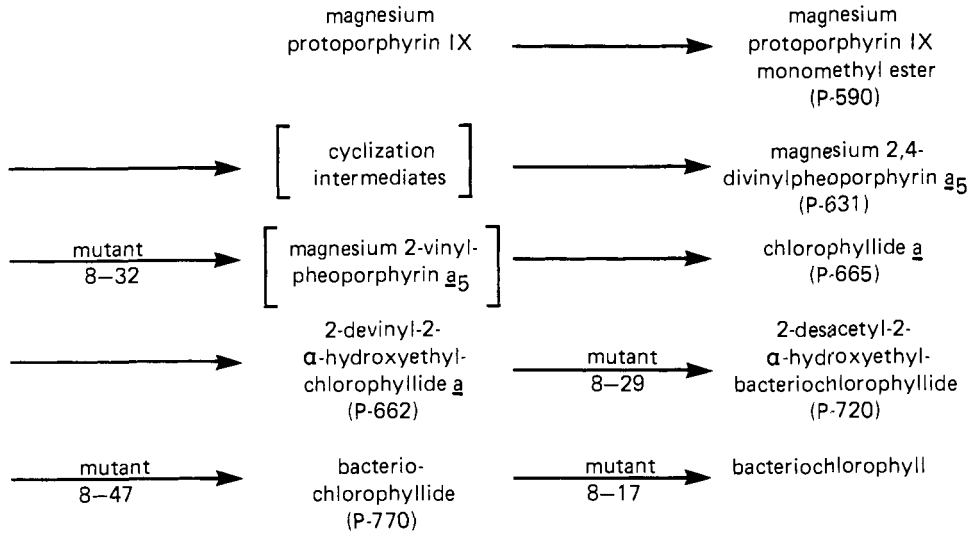

FIGURE 1: The magnesium branch of bacteriochlorophyll biosynthesis.

iopheophytin. They proposed that the latter complex might represent one of the light-harvesting forms of Bchl in the photosynthetic apparatus of *R. rubrum*. A summary of most of the known mutants of Bchl and chlorophyll *a* synthesis has recently appeared (Gough, 1972).

We have studied the pigment-protein complexes excreted by a number of Bchl-less mutants of *R. sphaeroides*. One of the pigments (P-730) excreted by a previously uncharacterized mutant (mutant 8) was purified and characterized as 2-desacetyl-2-vinylbacteriopheophorbide. The magnesium derivative of this compound (Figure 2) does not fit into the currently proposed biosynthetic pathway (Figure 1) and may thus indicate the existence of at least one alternate pathway for the synthesis of Bchl in *R. sphaeroides*.

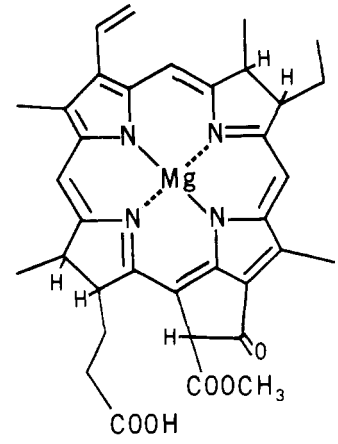

FIGURE 2: 2-Desacetyl-2-vinylbacteriochlorophyllide, the proposed structure for P-730 from mutant 8.

Experimental Procedures

Cultures and Maintenance. All mutants were derived from *Rhodopseudomonas sphaeroides* N.C.I.B. 8253 and were isolated and maintained on slope cultures as described by Lascelles (1966). The isolation of mutants 8-32, 8-29, 8-47, and 8-17 has been previously reported (Lascelles and Altshuler, 1967). Mutant 8 is a previously unreported mutant also isolated by Lascelles.

Growth and Incubation. The mutants were grown in 2-l. erlenmeyer flasks containing 1.0–1.5 l. of sterile medium MG (Lascelles, 1966). The flasks were shaken at 30° in the dark on a Burrell wrist-action shaker for 48 hr, or until the absorbance at 680 nm reached about 2.0. The cells were then harvested by centrifugation for 15 min at 5000*g*, and resuspended in half the volume of fresh medium A (Lascelles, 1966), but containing no Tween-80. This medium is referred to as "medium A^{-t}". The incubation was then continued for another 24–48 hr, or until the medium became visibly green. In later experiments a direct aseptic addition of 10 ml (per liter of medium MG) of a solution of 2.7 g of disodium succinate, 0.75 g of glycine, and 15 mg of L-methionine was made to the fully grown culture. This latter procedure had the advantage that the culture did not become contaminated and did not begin to decompose upon further incubation for 48–72 hr. After the incubation, the cells were removed by centrifugation for 15 min at 8000*g* and the green-colored supernatant was used for analysis of the pigments.

Analysis of the Pigments. The major pigments of mutants 8-32, 8-29, 8-47, and 8-17 have been previously determined (Richards and Lascelles, 1969). The pigment of mutant 8 was analyzed as follows: incubation of mutant 8 in medium A^{-t} produced a supernatant with red absorbance maxima at 730 and 670 nm in about a 1:1 ratio. The pigment responsible for the long-wavelength absorption was named P-730. The pigments were isolated by extracting the supernatants of medium A^{-t} at pH 5.5 with ethyl acetate as previously described (Richards and Lascelles, 1969). The ethyl acetate extract was evaporated and then partially purified by steady-state distribution in a 100-tube Quickfit Model 20 machine with a two-phase solvent system formed from petroleum ether, acetone, and water (2:2:1.1, v/v). After 50 upper and 50 lower phase transfers, the distribution was analyzed by measuring the absorption of both phases at 354 nm in a Bausch and Lomb Spectronic 20 spectrophotometer. The major fraction was located in tubes 35–55, with minor fractions located in tubes 1–5, 60–70, and 90–100. The ethyl acetate residue was also purified by polyethylene column chromatography. The major band was eluted with 80% methanol. This band was followed very closely by an incompletely separated secondary band. The procedures for derivative formation have been previously described (Richards and Lascelles, 1969) and include: oxidation with DDBQ (Lindsay-Smith and Calvin, 1966); hy-

Table I: Spectra of Fractions from Steady-State Distribution.

Fraction	Solvent	λ_{max} (nm)[a]								
Crude extract	Ethyl acetate	725 (1.00)	665 (0.58)	625 (0.45)		514 (0.69)	437 (4.22)	419 (4.41)	384 (2.91)	355 (3.66)
Fraction I (tube 2)	Aqueous acetone	720 (0.55)		600 (0.62)	560 (1.00)	523 (0.90)		410 (5.2)	396 (7.9)	350 (sh)
Fraction II (tube 45)	Petroleum ether–acetone	725 (0.90)	665 (1.00)	610 (0.20)	565 (0.18)	535 (0.18)	510 (0.90)	414 (3.2)	380 (3.0)	338 (5.5)
Fraction III (tube 65)	Petroleum ether–acetone	725 (0.14)	667 (1.00)	610 (0.18)		535 (0.12)	510 (0.25)	410 (2.3)		
Fraction IV (tube 95)	Petroleum ether–acetone	725 (0.50)	668 (1.00)				510 (1.6)	410 (5.9)		

[a] Relative absorption in parentheses.

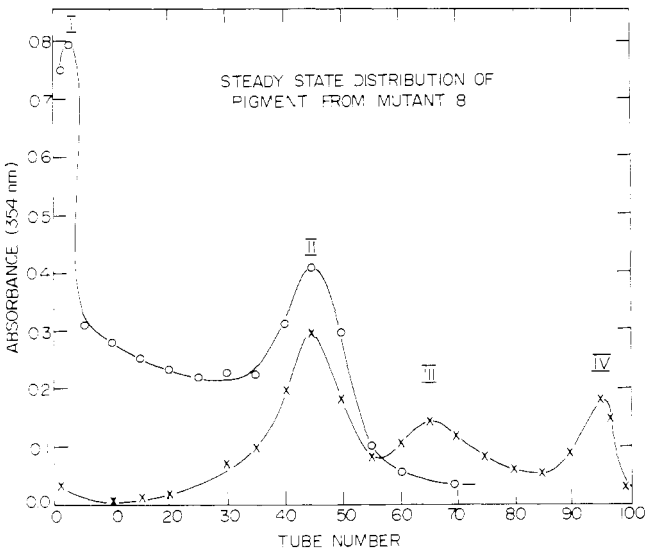

FIGURE 3: Steady-state distribution of pigments from mutant 8. The sample was introduced into tube 50. After 50 upper and 50 lower phase transfers, the absorbance of the lower (O) and upper (X) phases was measured at 354 nm.

dration by treatment with 30–32% HBr in acetic acid, followed by hydrolysis (Falk, 1964); and dehydration by heating to 240° in vacuo (Fischer et al., 1938). Kerosene paper chromatography (Chu et al., 1951; Barrett, 1959) was employed with Whatman No. 1 paper and a chloroform–kerosene (13:20, v/v) solvent system (with chloroform vapor equilibration). The development (ascending) was for 60 min (Richards and Lascelles, 1969). 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) at room temperature (Barrett, 1959).

Materials and Instrumental Measurements. All solvents, except kerosene, were distilled before use. The petroleum ether was a fraction with a boiling range of 65–110°. The kerosene (K-10) was from Fisher Scientific Co., Ltd., Montreal. The ether was washed with 10% (w/v) aqueous ferrous sulfate just before use to remove peroxides. All chemicals were of reagent grade. The 30–32% HBr in acetic acid was purchased from Eastman Kodak Co., Rochester, N.Y. The pigment, Ph-665 (pheophorbide *a*), was obtained from mutant 8-29 as previously described (Richards and Lascelles, 1969). Powdered polyethylene was obtained from Dow Chemical of Canada, Ltd., Vancouver. All visible spectra were recorded on a Cary Model 14 spectrophotometer.

Results

Table I shows the spectra of the ethyl acetate extract of the centrifuged incubation medium from mutant 8, both before and after fractionation by steady-state distribution. The spectrum of the crude extract indicated the presence of a tetrahydroporphyrin (Ph-730)[2] with absorption at 725, 514, 384, and 355 nm. It also indicated the presence of porphyrins, perhaps magnesium 2,4-divinylpheoporphyrin a_5 (P-631) with absorption at 625 and 437 nm, and magnesium protoporphyrin monomethyl ester (P-590) with absorption at 419 nm. The absorption at 665 nm may have been due to the presence of a small amount of a chlorin, pheophorbide *a* (Ph-665), although it was found that the tetrahydroporphyrin also has an absorption band at about the same wavelength.

The steady-state distribution fractionation is shown in Figure 3. The spectra of the fractions (Table I) indicated that magnesium had been lost from all of the pigments. Furthermore, Ph-730 (absorption at 720–725 nm) was found in all four fractions. Fraction I (tubes 1–5) contained predominantly porphyrins (presumably a mixture of Ph-631, its degradation products, and other more water-soluble porphyrins); fraction II (tubes 35–55) contained predominantly the tetrahydroporphyrin (Ph-730) plus some Ph-665; and fraction III (tubes 60–70) contained predominantly the chlorin (Ph-665) plus some Ph-730. Fraction IV (tubes 90–100) may have been a mixture of completely esterified pigments. The reason for incomplete separation of the Ph-730 from the Ph-665 may have been due to continual air oxidation of the tetrahydroporphyrin to the chlorin during the distribution.

The best separation of Ph-730 from Ph-665 was obtained with polyethylene column chromatography. Ph-730 was eluted with 80% methanol, although the yield was low. A spectrum of the polyethylene-purified Ph-730 is given in Table II and is also shown in Figure 4. This spectrum was taken after HCl treatment to assure the complete removal of magnesium. The spectrum was nearly identical with that of the dehydration product of 2-desacetyl-2-α-hydroxyethylbacteriopheophorbide (Ph-720) obtained from mutant 8-47 (Table II). The dehydration product of this compound should be the 2-vinyl derivative. Ph-730 was also treated with DDBQ, a procedure shown to convert tetrahydroporphyrins to chlorins (Lindsay-Smith and Calvin, 1966). The

[2] 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 tetrapyrrole excreted during incubations in medium A.

Table II: Spectra of Purified Ph-730 and Derivatives from Mutant 8.

Compound	λ_{max} (nm)[a]								
Ph-730[b]	725 (1.00)	667 (0.61)	608 (0.18)		512 (0.90)	480 (0.41)	405 (sh)	380 (1.73)	354 (2.02)
Ph-720 after dehydration in vacuo[c]	725 (1.00)	664 (0.56)	610 (0.17)		510 (0.72)	480 (0.24)		381 (2.40)	353 (2.87)
Ph-730 after DDBQ		668 (1.00)	605 (0.22)	565 (0.21)	530 (0.26)	504 (0.34)	409 (3.10)		
Pheophorbide *a*[d]		667 (1.00)	610 (0.15)	560 (0.06)	534 (0.21)	505 (0.23)	408 (2.09)		
Ph-730 after DDBQ, then hydration with HBr–acetic acid		659 (1.00)	599 (0.19)	558 (0.19)	528 (0.26)	500 (0.32)	407 (3.59)		
2-Devinyl-2-α-hydroxyethyl-pheophorbide *a*[e]		659 (1.00)	603 (0.13)	554 (0.07)	532 (0.20)	492 (0.20)	405 (2.22)		

[a] All spectra in ether; relative absorption in parentheses. [b] Purified by polyethylene chromatography. [c] Richards and Lascelles (1969). [d] Smith and Benitez (1955). [e] Jones (1964).

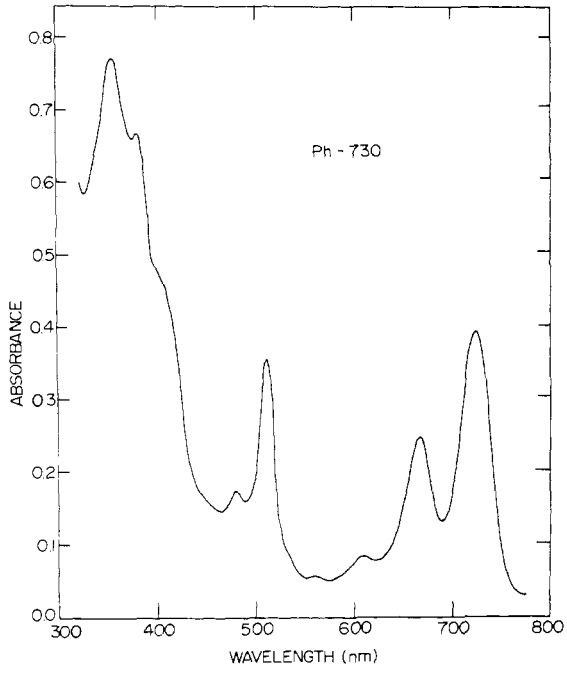

FIGURE 4: The spectrum, in ether, of 2-desacetyl-2-vinylbacteriopheophorbide (Ph-730) from mutant 8.

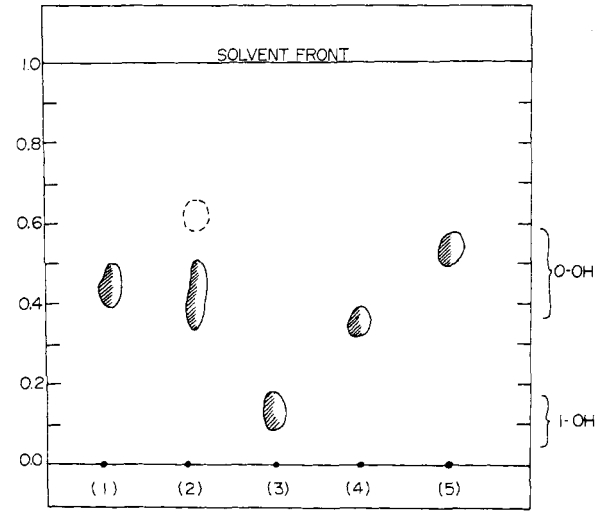

FIGURE 5: Kerosene paper chromatography of pigment methyl esters. Development was for 1 hr with chloroform–kerosene (13:20, v/v) in the presence of chloroform vapor. (1) Ph-730; (2) the chlorin produced from Ph-730 by DDBQ oxidation; (3) the hydration product of the chlorin; (4) the acetylation product of the hydrated chlorin; (5) pheophorbide *a* (Ph-665).

fraction was completely converted to a chlorin spectroscopically similar to pheophorbide *a* (Table II). A similar compound was also produced by treating Ph-720 with DDBQ followed by dehydration in vacuo, or vice versa (Richards and Lascelles, 1969). The presence of the vinyl group in the chlorin was confirmed by its hydration (treatment with HBr in acetic acid followed by hydrolysis). The spectrum of the product was similar to that of 2-devinyl-2-α-hydroxyethylpheophorbide *a* (Table II). The presence of the hydroxyethyl group in this product was confirmed by kerosene paper chromatography of the methyl esters (Figure 5). The hydration product had an R_F of 0.10, whereas that of Ph-730 and the DDBQ-produced chlorin had R_F's of about 0.45. Acetylation of the hydration product with acetic anhydride in pyridine resulted in a shift of the R_F to 0.35. The acetylation procedure did not affect the R_F of the DDBQ-produced chlorin (not shown), indicating one hydroxy group in the former and none in the latter (Figure 5).[3] These data are all consistent with the major pigment (Ph-730) being 2-desacetyl-2-vinylbacteriopheophorbide.

Discussion

The identification of the major pigment (Ph-730) excreted by *R sphaeroides* mutant 8 as 2-desacetyl-2-vinylbacteriopheophorbide presents a major difficulty for the proposed scheme of Bchl synthesis shown in Figure 1: the magnesium derivative of this compound is not on the proposed pathway. It is possible, however, that the enzymes involved in the magnesium branch of Bchl synthesis may catalyze the conversion of more than one substrate. This would allow the ex-

[3] The R_F values reported in this work are significantly lower than those reported earlier for this method (Richards and Lascelles, 1969), but similar to those reported by Barrett (1959). The method appears to be very sensitive to the presence of chloroform vapor, the larger R_F values being obtained only with adequate prior equilibration of chloroform vapor throughout the entire chromatography tank.

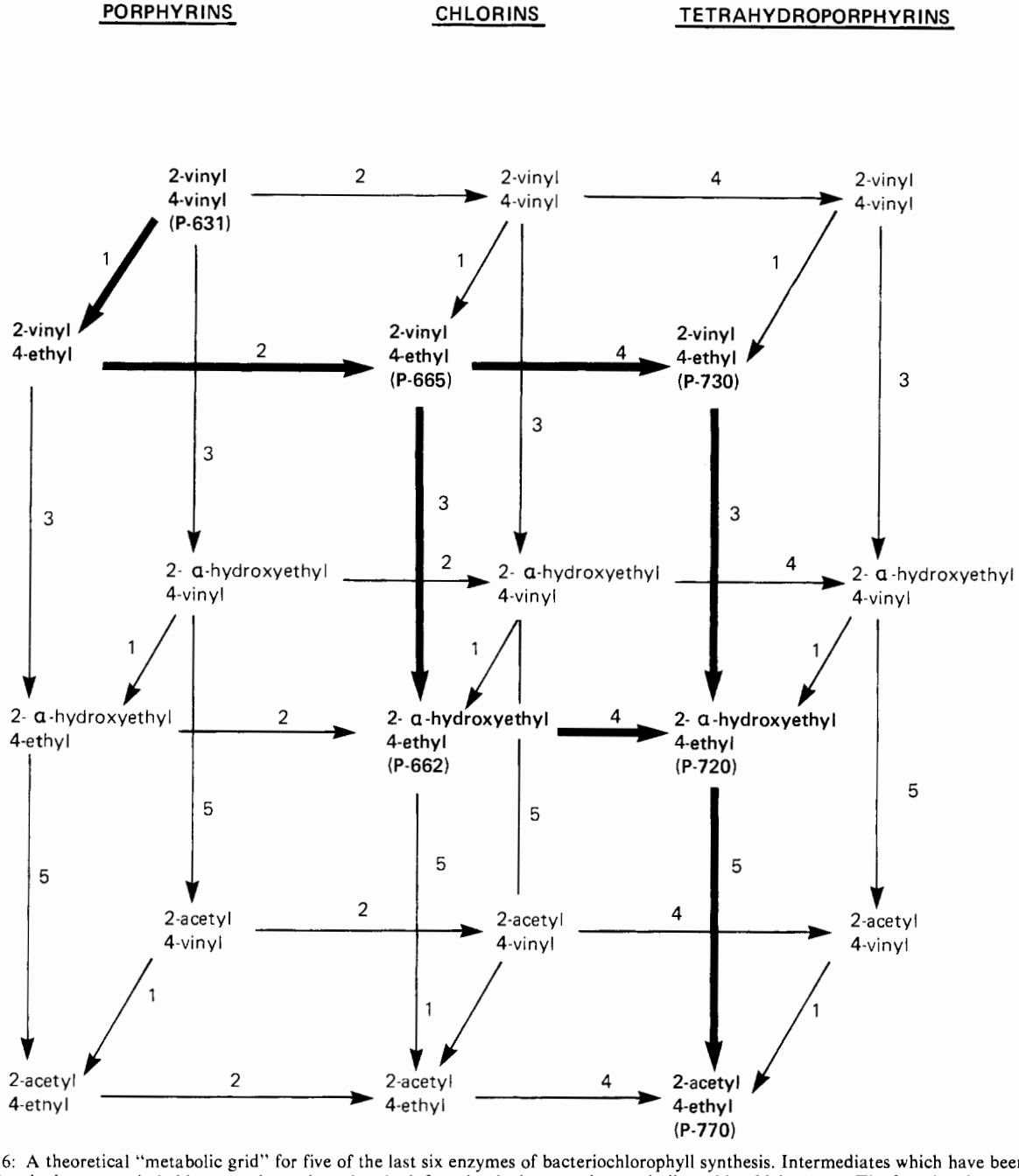

FIGURE 6: A theoretical "metabolic grid" for five of the last six enzymes of bacteriochlorophyll synthesis. Intermediates which have been isolated from *Athiorhodaceae* are in bold type and reactions thereby inferred to be in operation are indicated by thick arrows. The functional groups in the 2 and 4 positions are specified, and all intermediates are derivatives of the following compounds: porphyrins, magnesium pheoporphyrin a_5; chlorins, chlorophyllide *a*; tetrahydroporphyrins, bacteriochlorophyllide. See the text for a description of the enzymes involved (numbered 1–5 in the diagram).

istence of parallel pathways of Bchl synthesis. Such a "metabolic grid" is shown in Figure 6, for five of the last six enzymes involved in Bchl synthesis (after cyclization of the cyclopentenone ring). Parallel pathways have already been shown to be in operation in *Chlorella* during the latter stages of chlorophyll *a* synthesis (Ellsworth and Aronoff, 1969; Aronoff et al., 1971). In Figure 6, all of the compounds in the rear plane of the three-dimensional scheme retain the 4-vinyl group, while the 4-vinyl has been reduced to 4-ethyl for those compounds in the front plane. The functional group in the 2 position must be altered in the order: 2-vinyl → 2-α-hydroxyethyl → 2-acetyl. All three of these conversions could, theoretically at least, occur at any of the three stages of oxidation of the tetrapyrrole: porphyrin (left), chlorin (center), and tetrahydroporphyrin (right). The proposed final step in Figure 1, the phytylation reaction, could also theoretically occur with any of the intermediates listed in Figure 6, if the enzyme responsible were sufficiently nonspecific. The compounds in bold type in Figure 6 have all been detected in mutants of *Athiorhodaceae*, and hence the enzyme-catalyzed reactions designated with thick arrows have been inferred to be in operation in these mutants.

Mutant 8-32 of *R. sphaeroides* is presumably deficient in enzyme 1 (responsible for reduction of the 4-vinyl to the 4-ethyl group; Richards and Lascelles, 1969). In addition to P-631, some P-770 was also excreted, indicating that the mutation was "leaky" and that the mutant contained all of the other enzymes necessary for Bchl synthesis except the

phytylation enzyme. Since the major pigment accumlated was P-631, however, this compound is apparently not an effective substrate for either enzymes 2 or 3. Mutant Ala ·pho⁻ of *R. capsulata* is presumably deficient in enzyme 2 (responsible for reduction of the porphyrin to the chlorin) since it accumulated the phytyl ester of magnesium 2-vinylpheoporphyrin a_5 (Drews et al., 1971). This confirms that reduction of the 4-vinyl group can occur readily at the porphyrin stage, but does not determine whether this might occur before, during, or after the cyclization of the cyclopentenone ring. Mutant 8 of *R. sphaeroides* is presumably deficient in enzyme 3 (responsible for hydration of the 2-vinyl to the 2-α-hydroxyethyl group). This mutant does contain enzyme 4 (responsible for reduction of the chlorin to the tetrahydroporphyrin), however, since it accumulated both P-665 and P-730. This latter enzyme is presumably deficient in mutant 8-29 of *R. sphaeroides* which accumulated both P-662 and P-665 (Richards and Lascelles, 1969). This same study also showed that mutant 8-47 of *R. sphaeroides*, which is presumably deficient in enzyme 5 (responsible for oxidation of the 2-α-hydroxyethyl to the 2-acetyl group), accumulated all four of these intermediates in the relative amounts P-720 > P-662 > P-665 ≫ P-730. Hence, the results with the mutants indicate that at least two sequential orderings of the latter-stage enzymes of Bchl synthesis in *Athiorhodaceae* are possible: 1 → 2 → 3 → 4 → 5 and 1 → 2 → 4 → 3 → 5. Nothing definitive can be said, however, about the relative importance of all of the pathways shown in Figure 6 in the wild-type strains until studies at the enzyme level have been carried out. In addition, the question of whether the protein portion of the complex acts as a natural "carrier protein" necessary for Bchl synthesis will also have to await further enzymatic studies.

All of the intermediates so far detected in the *R. sphaeroides* mutants have been nonphytylated. This may be because phytol synthesis is impaired in all of these mutants or because it is the final step in the biosynthetic sequence. Mutant 8-17 of *R. sphaeroides* accumulated P-770, and was presumed to be deficient in the enzyme responsible for phytylation (Richards and Lascelles, 1969). However, it may be that this mutant, and others blocked at earlier stages, simply lack phytol. No phytol could be detected in mutants 8-32, 8-29, or 8-17, while it was easily detectable in the wild-type strain (Brown and Lascelles, 1972). Many of the same intermediates as those shown in Figure 1 have been detected as the phytyl esters in mutants of *R. palustris* (Krasnovskii et al., 1970) and *R. capsulata* (Drews et al., 1971). It would therefore appear likely that phytylation can occur at earlier stages in Bchl synthesis in at least some of the *Athiorhodaceae*.[4]

[4] It has recently been demonstrated that *R. rubrum* (and one other species out of 13 examined) is esterified with *all-trans*-geranylgeraniol rather than phytol (Künzler and Pfennig, 1973).

Acknowledgments

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