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Studies on the Structure of a Pigment Related to Chlorophyll *a* Produced by *Rhodopseudomonas spheroides*

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When *Rhodopseudomonas spheroides* is grown in a medium which contains 8-hydroxyquinoline, bacteriochlorophyll synthesis is partly inhibited and a number of pigments accumulate in the cells and medium (Jones, 1963*b*); these may be intermediates, or related to intermediates, in the biosynthesis of bacteriochlorophyll (Jones, 1963*c*). One of these compounds is magnesium 2,4-divinylphaeophorphyrin a_5 monomethyl ester (Jones, 1963*c*), and another is spectroscopically identical with phaeophorbid *a*, the magnesium-free derivative of chlorophyllide *a*.

One of the pigments (compound 2 of Jones, 1963*b*), which was isolated from the inhibited cells and medium, corresponds very closely in spectroscopic properties with the magnesium-free derivative of *Chlorobium* chlorophyll (650), the photosynthetic pigment of certain bacteria (Stanier & Smith, 1960). Compound 2 differs from *Chlorobium* phaeophorbid (650) in that it gives a positive reaction in the Molisch phase test for the presence of an isocyclic ring substituted with a carboxylic group, a reaction given by chlorophyll *a*, bacteriochlorophyll and the magnesium-free derivatives of these compounds (phaeophytins).

In the present paper are described studies on the structure of compound 2 which have led to its identification as 2-devinyl-2-hydroxyethylphaeophorbid *a*. Its possible significance in the biosynthesis of bacteriochlorophyll is discussed.

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MATERIALS AND METHODS

Hydrochloric acid solutions. To conform with the usual convention in this field concentrations of HCl are given as % (w/v).

Tetrapyrrole compounds. Phaeophorbid *a* was a gift from Dr J. E. Falk. 2-Devinyl-2-hydroxyethylmethylphaeophorbid *a* was a gift from Mr J. Barrett, Royal North Shore Hospital, Sydney, Australia. It was further purified by chromatography on a cellulose column (30 cm. × 2 cm.) equilibrated with light petroleum (b.p. 40–60°)–diethyl ether (10:1, v/v). The column was developed with light petroleum containing increasing concentrations of ether (cf. Morell, Barrett & Clezy, 1961). Protoporphyrin mono-

methyl ester was prepared as described by Jones (1963*a*). Compound 2 was prepared as described by Jones (1963*b*), except that only the pigments soluble between 8 and 15% HCl were chromatographed. This product had band ratios slightly different from those previously described (Jones, 1963*b*), although the positions of the bands were identical.

Oxidation of the hydroxylated side chains of tetrapyrrole compounds. The chromium trioxide–H_2SO_4 reagent of Bowden, Heilbron, Jones & Weedon (1946) was used, in acetone solution, as described by Barrett & Clezy (1959). The progress of the reaction was followed with the hand spectroscope; the reaction was judged complete when the band at about 658 mμ, characteristic of the hydroxyethylphaeophorbid, had shifted to about 680 mμ as expected for the acetyl derivative. Degradation products and unchanged phaeophorbid were washed out of ether solution with 15% HCl.

Dehydration of hydroxylated side chains. The toluene-*p*-sulphonic acid reagent was used in benzene solution as described by Clezy & Barrett (1961). When compound 2 was used the reaction products were poured into ether and then extracted into 25% HCl. The acid layer was adjusted to pH 4 with sodium acetate and extracted with ether. This ether layer was washed well with water and then 15% HCl, which removed any unconverted compound 2.

Esterification of carboxylic side chains. Ethereal solutions of the tetrapyrrole pigments were esterified with diazomethane.

Paper chromatography. Methylphaeophorbids were chromatographed by the method of Chu, Green & Chu (1951), chloroform–kerosene (paraffin) (2·6:4, v/v) and propan-1-ol–kerosene (1:5, v/v) being used as solvents. Free carboxyl groups in pigments were detected by chromatography in 2,6-lutidine–water (13:7, v/v) (cf. Falk, 1961).

Detection of hydroxylated side chains. The paper-chromatographic method of Barrett (1959) was used. This method makes use of the difference in R_F of porphyrins or chlorins before and after acetylation of hydroxyl groups in the side chain.

Molisch phase test. This reaction was carried out as described by Smith & Benitez (1955).

Determination of methoxyl groups. The method of Granick (1961) was used.

Spectra. Spectra were determined with a Bausch and Lomb Spectronic 505 recording spectrophotometer, calibrated by reference to a mercury emission spectrum.

RESULTS AND DISCUSSION

The spectroscopic properties of compound 2 closely resemble those of *Chlorobium* phaeophytin (650) and 2-devinyl-2-hydroxyethylphaeophorbid *a* (Table 1).

Both these compounds possess a hydroxyethyl substituent at position 2 of ring I of the phaeophorbid nucleus (Fig. 1). They differ at position 10 of ring V, where the phaeophorbid *a* derivative has a methoxycarbonyl substituent and the phaeophorbid (650) is unsubstituted. The latter material also has alkyl substituents of differing chain length at positions 4 and 5 (Holt, Hughes, Kende & Purdie, 1963). Compound 2 gave a positive Molisch phase test, which suggested that it possessed ring V intact, with the methoxycarbonyl group present, and that it was closely related to hydroxyethylphaeophorbid *a* in structure.

The presence of a hydroxylated side chain in compound 2 was shown by the method of Barrett (1959). The R_F of the compound on chromatography in the solvents of Chu *et al.* (1951) was greatly increased by acetylation (Table 2). The identity of R_F of compound 2 and its acetylated derivative with that of 2-devinyl-2-hydroxyethylphaeophorbid *a*, and its acetylated derivative suggests that compound 2 has the same alkyl substituents. This was also supported by the similarity of their hydrochloric acid numbers (Willstätter & Mieg, 1906). Each had a hydrochloric acid number of about 14.

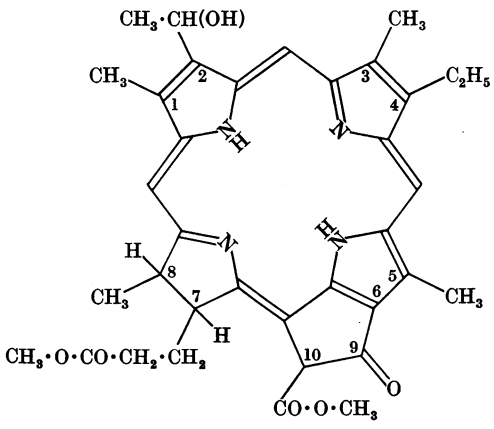
Oxidation of compound 2 by the method of Barrett & Clezy (1959) confirmed the presence of a hydroxyl substituent. The oxidation product had a maximum in the red region at 680 mμ, as would be expected after the formation of 2-acetylphaeophorbid *a* (Table 3), and supports the position of the α-hydroxyalkyl group of ring I (cf. Holt *et al.* 1963).

If compound 2 is 2-devinyl-2-hydroxyethylphaeophorbid *a*, dehydration of the hydroxylated

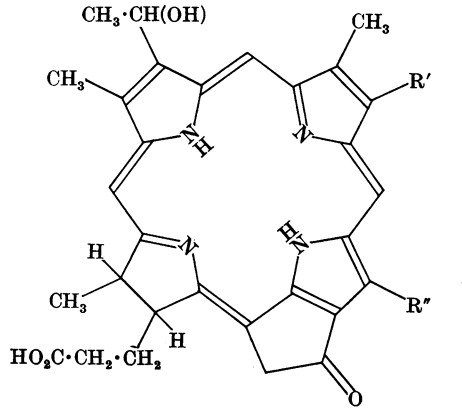
Table 1. *Absorption maxima and band ratios in ether of compound 2 and some related compounds*

Compound	Spectral properties						
Compound 2							
$\lambda_{max.}$ (mμ)	659	603	554	532	502	469	406
Band ratios	44·0	6·8	2·5	8·8	8·8	3·1	100
2-Devinyl-2-hydroxyethyl-phaeophorbid *a*							
$\lambda_{max.}$ (mμ)	659	603	554	532	592	469	405
Band ratios	44·8	6·4	2·9	9·1	9·4	3·8	100
Chlorobium phaeophytin (650) (Stanier & Smith, 1960)							
$\lambda_{max.}$ (mμ)	660	603	555	531	592	470	405
Band ratios	49·6	6·6	2·5	9·8	8·9	3·1	100

side chain should yield phaeophorbid *a*. In Table 3 the spectroscopic properties of the product of dehydration are compared with those of phaeophorbid *a*. It can be seen that the compounds are indeed very similar. This similarity extends also to chromatographic properties: fully esterified compound 2 after dehydration had R_F identical with methylphaeophorbid *a* in both chloroform–kerosene and propanol–kerosene. This also suggests that the alkyl substituents in compound 2 are the same as those in phaeophorbid *a*.



(*a*)



(*b*)

Fig. 1. (*a*) 2-Devinyl-2-hydroxyethylmethylphaeophorbid *a*. (*b*) *Chlorobium* phaeophorbid (650). R′:$-CH_2\cdot CH\cdot(CH_3)_2$, $-CH_2\cdot CH_2\cdot CH_3$ or $-C_2H_5$; R″:$-C_2H_5$ or $-CH_3$.

Fully esterified compound 2 has R_F in lutidine–water similar to methylphaeophorbid *a*. After complete hydrolysis in 15% hydrochloric acid each gave a compound of lower and similar R_F (Table 4). Compound 2 as extracted had intermediate R_F, similar to that of the monocarboxylic compound, protoporphyrin monomethyl ester. These results suggest that compound 2 possesses two carboxylic groups, one of which is esterified. The esterifying alcohol was identified as methanol by the method of Granick (1961). The weight of compound 2 taken was calculated by assuming that it had the same extinction coefficient at the Soret band as phaeophorbid *a*. The yield from 0·26 mg. of compound 2 was 10·2 μg. of methanol; assuming one methoxyl group per molecule the theoretical yield was 11·8 μg.

These results all suggest that compound 2 is 2-devinyl-2-hydroxyethylphaeophorbid *a*. Support for this suggestion was obtained when a mixture of 2-devinyl-2-hydroxyethylphaeophorbid *a* and compound 2 was chromatographed on polyethylene equilibrated with 70% acetone. The mixture could not be resolved by this method.

Table 2. *Chromatographic properties of hydroxylated methylphaeophorbids and their acetylated products*

Compound	Solvent*: Chloroform–kerosene	Solvent*: Propanol–kerosene
Compound 2	0·13	0·33
Compound 2 acetate	0·75	0·70
2-Devinyl-2-hydroxyethyl-phaeophorbid *a*	0·13	0·31
2-Devinyl-2-hydroxyethyl-phaeophorbid *a* acetate	0·78	0·72

* See Materials and Methods section.

Table 3. *Absorption maxima in ether of the oxidation product of compound 2 and of some related compounds*

Compound								
Oxidation product of compound 2	$\lambda_{max.}$ (mμ)	679	622	545	513			
Oxidation product of 2-devinyl-2-hydroxyethylphaeophorbid *a*	$\lambda_{max.}$ (mμ)	680	622	544	513			
2-Acetylphaeophorbid *a**	$\lambda_{max.}$ (mμ)	681	620	544	511			
Dehydration product of compound 2	$\lambda_{max.}$ (mμ)	666	608·5	560	533	505	470	409
	Band ratios	44	7·6	3·1	9·5	11·2	4·1	100
Phaeophorbid *a*†	$\lambda_{max.}$ (mμ)	667	609·5	560	534	505	471	408·5
	Band ratios	48·4	7·4	2·7	9·5	11·0	3·9	100

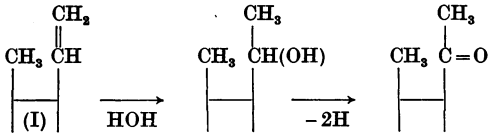
* Stern & Pruckner (1939).

† From Smith & Benitez (1955), with the assumption that the band ratios of phaeophytin *a* are the same as those of phaeophorbid *a*.

The suggested location of the methyl ester group at C-10 is based on analogy with bacteriochlorophyll and chlorophyll *a* (cf. Smith & Benitez, 1955), as is the location of methyl groups at positions 1, 3, 5 and 8 and the ethyl group at position 4.

A compound spectroscopically identical with phaeophorbid *a* and whose copper complex is identical with that of phaeophorbid *a* is also formed when *R. spheroides* is grown in the presence of 8-hydroxyquinoline (Jones, 1963*b*). It was possible that hydration of the vinyl group of this compound took place non-enzymically during the incubation, yielding compound 2. However, experiments where phaeophorbid *a* was added to growing cultures of *R. spheroides* did not result in any great increase in formation of compound 2. (A small amount of compound 2 is present in the medium of uninhibited cells.)

2-Devinyl-2-hydroxyethylchlorophyll *a* would be a logical intermediate between chlorophyll *a* and bacteriochlorophyll, which has an acetyl group at position 2 (Fig. 2). The latter is formed under anaerobic conditions and the conversion might be expected to involve successive hydration and dehydrogenation reactions at the vinyl group. Thus:



The finding of the magnesium-free pigment in cultures of 8-hydroxyquinoline-inhibited cells would be explained by the relative instability of magnesium complexes of the reduced porphyrins, such as phaeophorbids (Corwin & Wei, 1962). Bacteriochlorophyll itself is not found in the medium although the magnesium-free compound, bacteriophaeophytin, is found. Speculation that 2-devinyl-2-hydroxyethylphaeophorbid *a* is significant in bacteriochlorophyll synthesis is encouraged by the finding of a compound of similar spectroscopic properties in a blue–green mutant of *R. spheroides* (Sistrom, Griffiths & Stanier, 1956) and in a mutant of *R. spheroides* which is unable to synthesize bacteriochlorophyll (Griffiths, 1962). The pigment isolated by Sistrom *et al.* (1956) also gave a positive Molisch phase test but Griffiths (1962) does not report on this reaction.

Table 4. *Chromatographic properties of compound 2 compared with some model compounds*

The solvent was lutidine–water (see Materials and Methods section).

Compound	No. of free carboxyl groups	R_F
Compound 2	—	0·87
Compound 2, after esterification	—	0·94
Compound 2, after hydrolysis	—	0·82
Methylphaeophorbid *a*	0	0·93
Phaeophorbin *a*	2	0·81
Protoporphyrin monomethyl ester	1	0·88

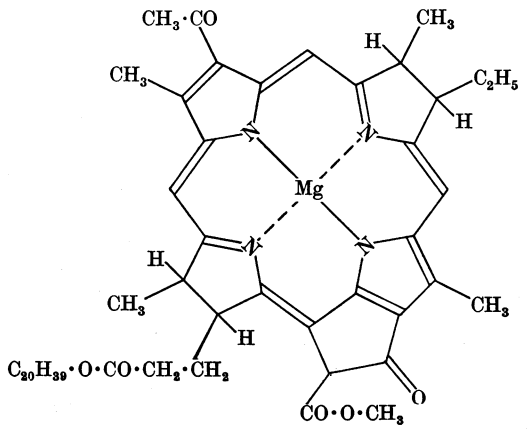


Fig. 2. Bacteriochlorophyll.

SUMMARY

1. A pigment (compound 2) isolated from cultures of *Rhodopseudomonas spheroides* grown in the presence of 8-hydroxyquinoline resembled *Chlorobium* phaeophorbid (650) and 2-devinyl-2-hydroxyethylphaeophorbid *a* in spectroscopic properties. Unlike *Chlorobium* phaeophorbid (650) it gave a positive phase test, indicating the presence of the isocyclic ring, with carboxymethyl substituent at C-10.

2. Chromatographic tests indicated that compound 2 possessed a hydroxylated side chain. This was confirmed by an oxidative reaction which yielded a product similar spectroscopically to 2-acetylphaeophorbid *a*.

3. Dehydration of compound 2 yielded a product which was virtually identical with phaeophorbid *a*, both spectroscopically and chromatographically. This strongly suggests that compound 2 is 2-devinyl-2-hydroxyethylphaeophorbid *a*. It was not separated from the latter compound by chromatography on polyethylene columns.

4. The presence of methanol as an esterifying group was confirmed by quantitative analysis.

5. It is suggested that compound 2 is closely related to an intermediate in the synthesis of bacteriochlorophyll.

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Metabolism of Amino Acids in the Lens

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The metabolism of amino acids in the lens has not been widely studied. This applies to the conversion of amino acids into other low-molecular-weight compounds, and also, to a lesser extent, to the incorporation of amino acids into proteins. Previous work on the metabolism of glycine (Merriam & Kinsey, 1950; Kinsey & Merriam, 1950) and of other amino acids (Dardenne & Kirsten, 1962) has been confined to rabbit lens; cattle lens was used in the work to be described since this was the species used previously in investigations of the metabolism of peptides (Cliffe & Waley, 1958) and of protein breakdown (Waley & van Heyningen, 1962; van Heyningen & Waley, 1963). Intact ox lenses have been incubated in solutions containing radioactive glycine (a metabolically active amino acid) and radioactive valine (a relatively unreactive amino acid), and the products have been separated by chromatography and electrophoresis, and detected by radioautography, in order to establish the main metabolic pathways involving these amino acids.

The kinetics of the incorporation of a mixture of radioactive amino acids into the proteins of ox lens has been studied in an attempt to assess the relative importance of turnover and net synthesis. The results suggest that the synthesis of proteins is more rapid than can be accounted for by growth, so that turnover is the main process responsible for the incorporation. An (unsuccessful) attempt was also made to detect low-molecular-weight intermediates in the synthesis of proteins.

EXPERIMENTAL

Materials

The radioactive amino acids (The Radiochemical Centre, Amersham, Bucks.) used were: [2-^{14}C]glycine, specific activity 5·69 μc/μmole, uniformly labelled L-[U-^{14}C]valine, specific activity 25·9 μc/μmole, and a hydrolysate (specific activity about 200 μc/mg.) of protein from *Chlorella vulgaris* grown on $^{14}CO_2$. There were five radioactive contaminants in the glycine, each containing about 0·3% of the total radioactivity; the contaminants were detected by electrophoresis and chromatography on paper of about 0·5 μc. The purity of the valine was comparable.

Methods

Media for incubations. Medium A contained (in 100 ml.): 0·25 ml. of 6·9% (w/v) $MgSO_4,7H_2O$, 0·5 ml. of 5·1% (w/v) $CaCl_2$, 1 ml. of 4·1% (w/v) KCl, 0·06 ml. of M-potassium phosphate buffer, pH 7·4, 10 ml. of 6·13% (w/v) NaCl, 10 ml. of 2·52% (w/v) $NaHCO_3$ (gassed with CO_2), 0·1 g. of glucose, 0·01 g. of penicillin, 0·01 g. of streptomycin and 0·01 g. of phenol red. Medium B contained, in 7 ml.: 55 mg. of 'TC medium dried 199' (Difco Laboratories, Detroit, Mich., U.S.A.), 1·0 ml. of 2·5% (w/v) $NaHCO_3$, 0·7 mg. of penicillin, 0·7 mg. of streptomycin and 7 mg. of glucose. A volume of 5 ml. of medium A or of medium B was used for each incubation. The incubation tubes were gassed with air–CO_2 (95:5) for 10 min., and then stoppered; they were rocked gently during the incubations, which were carried out at 34°.

Gel filtration. This was carried out on columns, 2·7 cm. × 15 cm. long or 2·75 cm. × 30 cm. long, of Sephadex G-25 (medium grade) at room temperature. The columns were equilibrated with the buffers used for elution, usually either