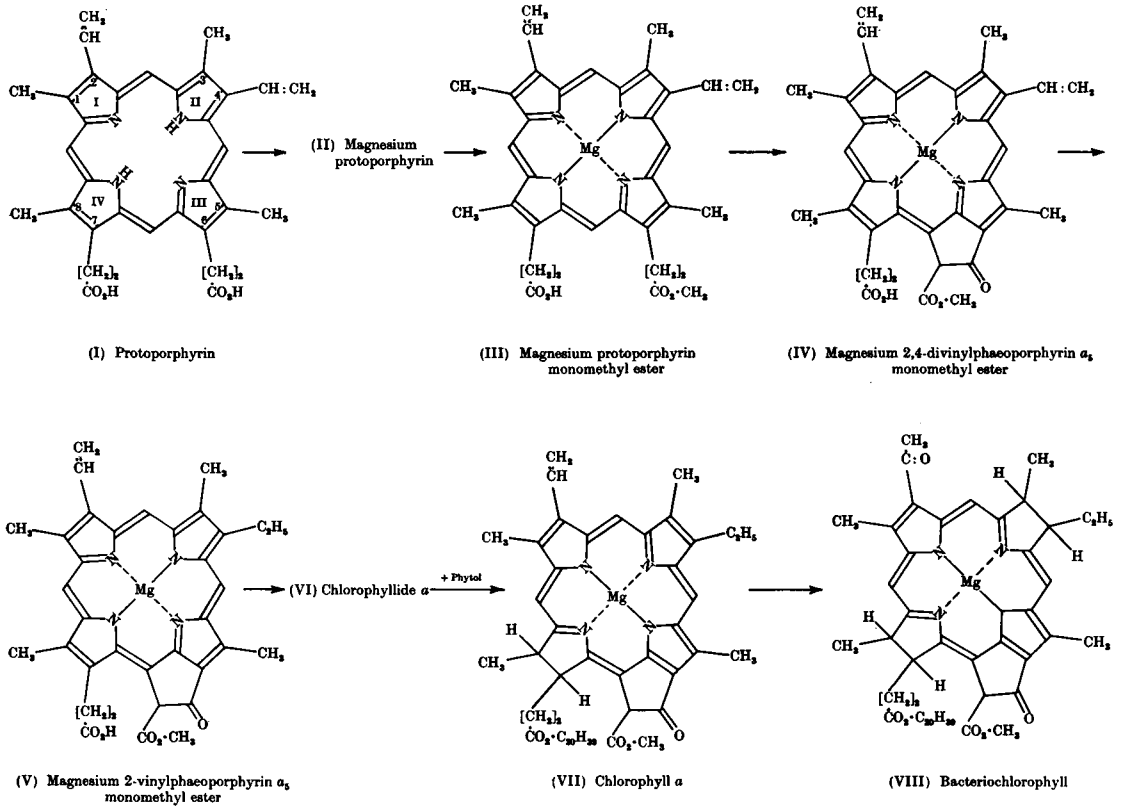
# Magnesium 2,4-Divinylphaeoporphyrin $a_5$ Monomethyl Ester, a Protochlorophyll-like Pigment Produced by *Rhodopseudomonas spheroides*

By O. T. G. JONES
*C.S.I.R.O., Division of Plant Industry, Canberra, Australia*

(*Received* 25 *March* 1963)

The identification of intermediates in the biosynthesis of chlorophyll has been largely dependent upon the use of mutants of *Chlorella* that are unable to synthesize chlorophyll but accumulate in the medium various tetrapyrrole compounds assumed to be precursors (for a review see Granick & Mauzerall, 1961). In particular, Granick (1950) identified magnesium vinylphaeoporphyrin $a_5$ monomethyl ester (Scheme 1, formula V) as an intermediate and it now seems likely that it is this compound and not its phytyl ester (protochlorophyll) that is reduced to the oxidation level of chlorophyll. Esterification with phytol follows, yielding chlorophyll *a* (formula VII) (cf. Smith, 1960). The detection in *Rhodopseudomonas spheroides* of an enzyme system which methylates magnesium protoporphyrin (formula II) (Tait & Gibson, 1961) and the isolation of magnesium protoporphyrin monomethyl ester (formula III) as a normal metabolite of *R. spheroides* (Jones, 1963*a*) indicated that the pathways of chlorophyll biosynthesis in green plants and bacteriochlorophyll synthesis in photosynthetic bacteria may be similar, although chlorophyll *a* and bacteriochlorophyll differ not only in the reduction state of ring II but also in substituents at position 2 of the tetrapyrrole




(I) Protoporphyrin
(III) Magnesium protoporphyrin monomethyl ester
(IV) Magnesium 2,4-divinylphaeoporphyrin $a_5$ monomethyl ester
(V) Magnesium 2-vinylphaeoporphyrin $a_5$ monomethyl ester
(VII) Chlorophyll *a*
(VIII) Bacteriochlorophyll

Scheme 1. Biosynthesis of bacteriochlorophyll.

nucleus (Scheme 1). In bacteriochlorophyll there is an acetyl group at position 2, whereas in chlorophyll *a* there is a vinyl group.

Stanier & Smith (1959) and Griffiths (1962) have described mutants of *R. spheroides* that are unable to synthesize bacteriochlorophyll but which accumulate a compound they have called bacterial protochlorophyll: it resembles plant protochlorophyll in spectroscopic properties, although differing slightly from it in the positions of the absorption maxima. When *R. spheroides* is grown in the presence of 8-hydroxyquinoline a number of pigments related to chlorophyll accumulate in both the cells and the medium (Jones, 1963*b*). One of these compounds (compound 5) appeared to be the same as that called bacterial protochlorophyll by Stanier & Smith (1959). Treatment of this pigment with acid led to formation of compound 7, believed to be a metal-free porphyrin.

In this paper are described studies on compounds 5 and 7 that have led to the identification of compound 5 as magnesium 2,4-divinylphaeoporphyrin $a_5$ monomethyl ester. Its possible role in chlorophyll biosynthesis is discussed.

## MATERIALS AND METHODS

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

*Polyethylene.* The powdered polyethylene used in chromatography (melt index 3·5) was a gift from the Dow Chemical Co., Mich., U.S.A.

*Tetrapyrrole compounds.* Monohydroxyethylmonovinyldeuteroporphyrin IX was a gift from Mr J. Barrett, Royal North Shore Hospital, Sydney, Australia; 2,4-diacetyldeuterohaem was a gift from Dr J. E. Falk. Protohaem was prepared from ox blood by the method of Labbe & Nishida (1957).

Protoporphyrin was prepared from crystalline protohaem by the method of Morell & Stewart (1956) (cf. Porra & Jones, 1963). 2,4-Diacetyldeuteroporphyrin was prepared from the corresponding haem by the method of Morell & Stewart (1956). After washing the ether solution with 5% HCl the diacetylporphyrin was extracted into 15% HCl; this extract was then neutralized and the porphyrin re-extracted into ether. Its spectrum was identical with that described by Lemberg & Falk (1951) for 2,4-diacetyldeuteroporphyrin.

Vinylphaeoporphyrin $a_5$ monomethyl ester (formula V; lacking magnesium) was prepared from etiolated bean plants (*Phaseolus vulgaris*). Cotyledons (200 g. wet wt.) were collected from dark-grown beans 10 days after planting and extracted with 2 l. of acetone. The yellow extract was transferred to ether, which was washed with 5% HCl and then shaken with 15% HCl. This removed magnesium from the metalloporphyrin and extracted the porphyrin. The 15% HCl fraction was adjusted to pH 4·0 with sodium acetate and extracted with ether, which was then washed with water until free of acetic acid. The ethereal solution was evaporated to dryness and the residue, dissolved in a small quantity of 70% (v/v) acetone, was placed on a column (2 cm. diam.) containing 20 g. of polyethylene (Anderson & Calvin, 1962) equilibrated with 70% (v/v) acetone, and the column was developed with 70% (v/v) acetone. Only one yellow band developed which, on elution, gave a spectrum in ether corresponding to that of protophaeophytin (the phytyl ester of vinylphaeoporphyrin $a_5$ methyl ester; Koski & Smith, 1948). The solubility of the material in 15% HCl indicated, however, that it was not a phytyl ester but vinylphaeoporphyrin $a_5$ monomethyl ester. This would be expected since the major protochlorophyll pigment in etiolated plants lacks the phytyl side chain (cf. Smith, 1960).

*Preparation of compound 5 and compound 7.* The metalloporphyrin whose structure was to be investigated was prepared as previously described (compound 5 of Jones, 1963*b*) by chromatography of the ether-soluble compounds from the medium of *R. spheroides* grown in the presence of 8-hydroxyquinoline. The metal-free pigment (compound 7 of Jones, 1963*b*) was prepared from the ether extract of medium by chromatography of the 15% HCl fraction on polyethylene as described above for the preparation of vinylphaeoporphyrin $a_5$ monomethyl ester.

*Preparation of porphyrin oximes (cf. Lemberg & Falk,* 1951). Oximes of porphyrins containing carbonyl groups were prepared by dissolving the porphyrin in pyridine, adding an excess of a mixture of equivalent amounts of solid hydroxylamine hydrochloride and $Na_2CO_3$ and refluxing for 20 min. This solution was cooled, ether was added and the pyridine was washed out with water.

*Formation of diazoacetic ester adducts of porphyrins (cf. Parker,* 1959). Excess of diazoacetic ester in ether solution was added to a small tube containing an ethereal solution of the vinyl-substituted porphyrin. The tube was flushed with $N_2$, heated to 60°, then stoppered and incubated at 60° for 20 hr. After cooling, the mixture was dissolved in ether and the porphyrin–diazoacetic ester adduct extracted with 15% HCl. After adjustment to pH 4 with sodium acetate it was taken back into ether.

*Hydrogenation of vinyl side chains.* Unsaturated side chains of various porphyrins were hydrogenated in acetic acid solution by using platinum black as catalyst (Warburg & Gewitz, 1951). The reduction was carried out at 40° for 40 min. Ether and water were then added, the mixture was shaken well in a separating funnel to oxidize any reduced porphyrin, and the ether solution washed with water until free of acid. After the hydrogenation of vinylphaeoporphyrins the products extracted from ether between 6 and 10% HCl were collected and transferred to ether at pH 4.

*Hydration of vinyl-substituted porphyrins (cf. Clezy & Barrett,* 1961). The porphyrin was treated with acetic acid containing 50% (w/v) of HBr. After 16 hr. 5% HCl was added and the hydrated porphyrin was extracted into ether at pH 4.

*Esterification of porphyrins.* Esterification of the carboxyl groups of the porphyrin side chains was carried out in ethereal solution with diazomethane.

*Paper chromatography.* Porphyrin methyl esters were chromatographed by the method of Chu, Green & Chu (1951) with chloroform–kerosene ('paraffin') (13:20, v/v) as solvent. This system was used in testing for hydroxyl groups on the side chains of porphyrins by the method of Barrett (1959), who made use of the difference in $R_F$ of the hydroxylated porphyrins before and after acetylation.

The 2,6-lutidine–water (13:7, v/v) solvent (cf. Falk, 1961) was used to determine by paper chromatography the number of free carboxyl groups on the porphyrin side chains. A mixture of protoporphyrin and its mono- and di-methyl esters, obtained by partial hydrolysis of protoporphyrin dimethyl ester (Jones, 1963*a*), was run as a marker.

*Test for methoxyl groups.* The chromotropic colour reagent of Feigl (1960) was used, as described by Jones (1963*a*).

*Formation of chloroporphyrin derivatives of phaeoporphyrins.* The method of Granick (1950) was employed. Phaeoporphyrins were allowed to stand overnight in methanol containing 30% (w/v) of dry HCl.

*Magnesium determination.* Magnesium was determined by atomic-absorption spectrophotometry (David, 1960). This method is sensitive to 0·05 p.p.m. of magnesium.

*Spectra.* A Bausch and Lomb spectronic 505 recording spectrophotometer, calibrated with respect to a mercury-emission spectrum, was used in determining u.v. and visible spectra. A Perkin–Elmer 237 spectrophotometer was used to record infrared spectra.

## RESULTS

### *Magnesium content of compound 5*

Compound 5 was similar in spectroscopic and solubility properties to magnesium vinylphaeoporphyrin $a_5$ methyl ester (formula V) and was readily converted by acid into compound 7, resembling vinylphaeoporphyrin $a_5$ methyl ester (Jones, 1963*b*). It was likely therefore that compound 5 was itself a magnesium complex. This was confirmed as follows.

An ethereal solution of compound 5 (300 ml.) was shaken with 5% HCl until the spectrum in the ether layer had completely changed to that of compound 7. The acid layer was collected, the ether washed with water until free of acid and the washings were added to the acid layer. The volume of the ether layer was adjusted to 300 ml. with washed ether and $E^{1\,cm.}_{421\cdot5\,m\mu}$ was measured. On the assumption that the $\epsilon_{mM}$ at the Soret maximum was 193, equal to that of vinylphaeoporphyrin $a_5$ (Granick, 1950), it was calculated that the ether solution contained 1·62 $\mu$moles of porphyrin. An equivalent amount of the magnesium complex should contain 39·3 $\mu$g. of magnesium; the atomic-absorption spectroscopic assay revealed the presence of 44 $\mu$g. of magnesium in the bulked acid extract and washings.

### *Deductions from the spectrum of the metal-free porphyrin, compound 7*

In Table 1 the spectroscopic properties of the porphyrin, compound 7, are given, together with those of some known compounds. Compared with vinylphaeoporphyrin $a_5$ the band maxima are shifted about 4 m$\mu$ to longer wavelengths; this is consistent with the effect of a second vinyl substituent on the porphyrin nucleus (cf. Lemberg & Legge, 1949). Such a shift might also be obtained if an acetyl group were substituted for the vinyl at position 2 (Fischer & Stern, 1940). In the latter case the ratio of absorptions band III:band IV should be higher than in vinylphaeoporphyrin $a_5$, since the effect of increasing the electrophilic nature of the substituents in the pyrrole ring opposite the strongly-electrophilic isocyclic-ring carbonyl group increases this ratio (Lemberg, 1953) (see ratios band III:band IV for phaeoporphyrin $a_5$ and vinylphaeoporphyrin $a_5$). Compound 7 does not show a high ratio. In fact the similarity of the ratios in compound 7 and phaeoporphyrin $a_5$, coupled with the shift to longer wavelength in the former, suggests that a second electrophilic group, possibly a vinyl group, has been substituted in the pyrrole ring (ring II) adjacent to the ring carrying the vinyl group (ring I). Two rhodofying groups (i.e. groups that increase the ratio band III:band IV) on vicinal rings are known to nullify each other's rhodofying effect (cf. Lemberg & Falk, 1951; Lemberg, 1953), although their effects upon wavelength of absorption are additive. Since the structure of other compounds in the chlorophyll series is based upon the phaeoporphyrin $a_5$ nucleus (see formulae IX) a likely structure for compound 7 is 2,4-divinylphaeoporphyrin $a_5$ monomethyl ester (Scheme 1 : IV, without magnesium). This structure was supported by the following evidence.

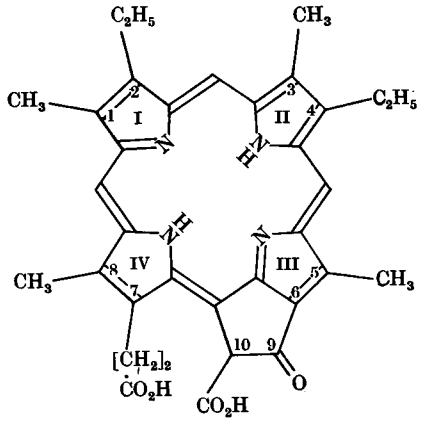
Table 1. *Absorption spectra of compound 7 and some related compounds*

All the compounds were in dioxan except the 2-acetylphaeoporphyrin $a_5$ dimethyl ester, which was in pyridine–ether.

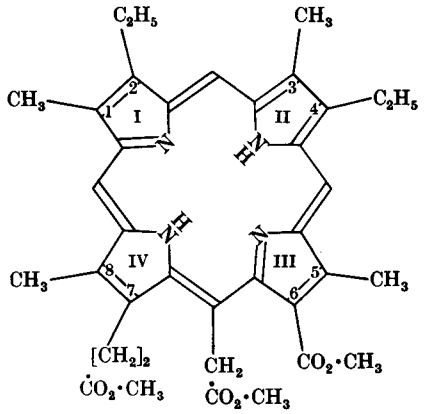
| | Absorption maxima (m$\mu$) | | | | |
|---|---|---|---|---|---|
| Band ... ... ... ... ... ... ... ... ... ... | I | II | III | IV | Band III/band IV |
| Phaeoporphyrin $a_5$ monomethyl ester* | 634 | 583 | 562 | 521 | 1·71 |
| Vinylphaeoporphyrin $a_5$ monomethyl ester | 638 | 587 | 567 | 524 | 2·1 |
| Compound 7 | 644 | 592 | 569 | 528 | 1·63 |
| 2-Acetylphaeoporphyrin $a_5$ dimethyl ester† | 645·5 | 596·5 | 571 | 527 | — |

* From Stern & Wenderlein (1935).
† From Fischer & Stern (1940).

*Infrared spectroscopy of compound* 7. Chlorophyll pigments and their derivatives have well-defined infrared spectra with a characteristic band in the 1690–1700 cm.$^{-1}$ region given by the isocyclic-ring carbonyl group (Holt & Jacobs, 1955). A formyl or acetyl group causes strong absorption in the 1660 cm.$^{-1}$ region, and carboxylic ester groups at about 1730 cm.$^{-1}$. Strong bands were found at 1700 cm.$^{-1}$, confirming the presence of the isocyclic ring, and at 1730 cm.$^{-1}$, showing an ester group. No band was found in the 1660 cm.$^{-1}$ region, indicating that no acetyl substituent was present.




(IX) Phaeoporphyrin $a_5$




(X) Chloroporphyrin $e_6$ trimethyl ester

*Evidence for the presence of only one carbonyl group in compound* 7. The magnitude of the shift of absorption bands to shorter wavelengths when an oxime is formed can be taken as a measure of the number of carbonyl groups conjugated to the porphyrin molecule. This shift is greater for the oxime of an isocyclic-ring carbonyl group than for the oxime of an acetyl carbonyl group (cf. Lemberg & Falk, 1951). From Table 2 it can be seen that the oxime shift observed with compound 7 is of the same order as that found for other monocarbonyl phaeoporphyrins and smaller than that found for the dicarbonyl 2,4-diacetyldeuteroporphyrin or 2-acetylphaeoporphyrin $a_5$ (Fischer & Stern, 1940). This suggests that only one carbonyl group, that of the isocyclic ring, is conjugated with the porphyrin-ring system and that compound 7 cannot be 2-acetylphaeoporphyrin $a_5$.

*Evidence for the presence of two vinyl groups in compound* 7. Porphyrins with vinyl substituents form adducts with diazoacetic ester that have spectra with bands shifted to shorter wavelengths (Fischer & Stern, 1940). With compound 7 a shift in band maxima is observed that is greater than that obtained with vinylphaeoporphyrin $a_5$ and similar to that for the divinylporphyrin, protoporphyrin (Table 3). Thus two vinyl groups are probably present in compound 7.

Under the conditions for hydrogenation described in the Materials and Methods section the isocyclic-ring carbonyl is not reduced and the expected product of the reduction of both compound 7, if this is indeed divinylphaeoporphyrin $a_5$, and of monovinylphaeoporphyrin $a_5$ is phaeoporphyrin $a_5$. The spectra of the products of hydrogenation are given in Table 4. The shift in the spectrum on hydrogenation of compound 7 is consistent with the reduction

Table 2. *Absorption spectra of porphyrin oximes*

All the compounds were in dioxan except the 2-acetylphaeoporphyrin $a_5$ dimethyl ester oxime, which was in pyridine–ether. Δ refers to the average shift of the four bands when the oxime is formed.

| | No. of carbonyl groups | Absorption maxima (mμ) Band I | Band II | Band III | Band IV | Δ (mμ) |
|---|---|---|---|---|---|---|
| Phaeoporphyrin $a_5$ monomethyl ester oxime* | 1 | 625 | 573 | 550 | 512 | 10 |
| Vinylphaeoporphyrin $a_5$ monomethyl ester oxime | 1 | 628 | 575 | 555 | 515 | 10·7 |
| Compound 7 oxime | — | 633 | 580 | 556 | 519 | 11·2 |
| 2-Acetylphaeoporphyrin $a_5$ dimethyl ester oxime† | 2 | 629·6 | 576 | 552·6 | 516 | 16·1 |
| 2,4-Diacetyldeuteroporphyrin dimethyl ester oxime | 2 | 625 | 576 | 537 | 503 | 13·2 |

* From Stern & Wenderlein (1936).
† From Fischer & Stern (1940).

of two vinyl groups and with the formation of phaeoporphyrin $a_5$. A similar shift in band position to shorter wavelength was found on the hydrogenation of protoporphyrin ester, but the carbonyl compound, diacetyldeuteroporphyrin, was not reduced under these conditions.

*Reactions of the chloroporphyrin derivatives of compound 7.* Porphyrins containing an isocyclic ring (phaeoporphyrins) undergo a characteristic methanolysis when treated with anhydrous hydrochloric acid in methanol, resulting in splitting of the isocyclic ring and formation of chloroporphyrin methyl ester (Fischer & Stern, 1940) (formulae IX and X). Thus phaeoporphyrin $a_5$ gives rise to chloroporphyrin $e_6$ (Granick, 1950). The corresponding derivative of compound 7 and of vinylphaeoporphyrin $a_5$ was prepared as described in the Materials and Methods section. In Table 5 are given details of the spectra of the chloroporphyrin derived from compound 7 and of some related compounds. The shift of the absorption maxima to longer wavelengths, as well as the ratio band III: band IV suggests that the chloroporphyrin derivative of compound 7 is a divinyl derivative of chloroporphyrin $e_6$. On hydrogenation of the vinyl substituents both vinylchloroporphyrin $e_6$ and the chloroporphyrin derived from compound 7 gave a product closely resembling chloroporphyrin $e_6$ (Table 5).

*Chromatographic evidence for the presence of two vinyl groups in the chloroporphyrin derived from compound 7.* The chloroporphyrin was hydrated as described by Clezy & Barrett (1961). This procedure converts vinyl groups into α-hydroxyethyl groups, so that protoporphyrin (formula I), for example, is converted into haematoporphyrin. The hydrated porphyrin was transferred to ether at pH 4, the ether solution washed with 0·36% hydrochloric acid and the product extracted with 1% hydrochloric acid. The extract was adjusted to pH 4 and the porphyrin transferred to ether. The spectrum of the porphyrin resembled that of

Table 3. *Effect of diazoacetic ester on the absorption maxima of some vinyl porphyrins*

The solvent was ether. Δ refers to the average shift of the four bands.

| | No. of vinyl groups | Absorption maxima of porphyrin (mμ) | | | | Absorption maxima of diazoacetic ester adduct (mμ) | | | | Δ (mμ) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Band I | Band II | Band III | Band IV | Band I | Band II | Band III | Band IV | |
| Vinyl phaeoporphyrin $a_5$ monomethyl ester | 1 | 638 | 586 | 566 | 524 | 636 | 583 | 564 | 523 | 2 |
| Protoporphyrin | 2 | 633 | 576 | 536 | 503 | 627 | 572 | 532 | 501 | 4·5 |
| Compound 7 | | 643 | 590 | 567 | 527 | 639 | 585 | 563 | 525 | 3·8 |

Table 4. *Effect of hydrogenation on the spectra of some vinyl-substituted porphyrins*

The solvent was ether. Δ refers to mean shift of the four bands after hydrogenation.

| | Absorption maxima of the hydrogenated derivative (mμ) | | | | Δ (mμ) |
|---|---|---|---|---|---|
| | Band I | Band II | Band III | Band IV | |
| Vinylphaeoporphyrin $a_5$ monomethyl ester | 634 | 581 | 562 | 520 | 4·25 |
| Protoporphyrin | 623 | 567 | 526 | 497 | 8·7 |
| Compound 7 | 635 | 581 | 562 | 520 | 7·5 |
| Diacetyldeuteroporphyrin dimethyl ester | 639 | 586 | 546 | 512 | 0 |

Table 5. *Absorption maxima in dioxan of the chlorophyrins derived from compound 7 and related porphyrins and of the hydrogenated chloroporphyrins*

| | Absorption maxima (mμ) | | | | |
|---|---|---|---|---|---|
| | Band I | Band II | Band III | Band IV | Band III/ band IV |
| Chloroporphyrin $e_6$ trimethyl ester* | 629 | 576 | 543 | 506 | 0·64 |
| Vinylchloroporphyrin $e_6$ trimethyl ester | 633 | 579 | 548 | 511 | 0·87 |
| Chloroporphyrin from compound 7 | 639 | 585 | 551 | 514 | 0·62 |
| Hydrogenated vinylchloroporphyrin $e_6$ trimethyl ester | 630 | 576 | 544 | 506 | 0·68 |
| Hydrogenated chloroporphyrin from compound 7 | 630 | 576 | 545 | 507 | 0·62 |

* From Stern & Wenderlein (1935).

Table 6. *$R_F$ values of some hydroxylated porphyrins and their acetylated products*

The method used was described by Barrett (1959). Chloroform–kerosene (13:20, v/v) was used as solvent.

| Compound (methyl ester) | $R_F$ | | No. of OH groups |
|---|---|---|---|
| | Hydroxylated porphyrin | Acetate of hydroxylated porphyrin | |
| Hydroxylated chloroporphyrin from compound 7 | 0 | 0·76 | |
| Haematoporphyrin | 0 | 0·79 | 2 |
| Chloroporphyrin from compound 7 | 0·78 | 0·78 | |
| Hydroxylated vinylchloroporphyrin $e_6$ | 0·27 | 0·71 | 1 |
| Monohydroxyethylmonovinyl deuteroporphyrin | 0·4 | 0·73 | 1 |

Table 7. *$R_F$ values of compound 7 before and after hydrolysis compared with the chromatographic behaviour of some model porphyrin esters*

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

| Porphyrin | No. of free carboxyl groups | $R_F$ |
|---|---|---|
| Compound 7 | — | 0·82 |
| Compound 7, after hydrolysis | — | 0·68 |
| Protoporphyrin dimethyl ester | 0 | 0·93 |
| Protoporphyrin | 2 | 0·7 |
| Partly hydrolysed protoporphyrin dimethyl ester* | 0, 1 and 2 | 0·94, 0·85, 0·7 |
| Mesoporphyrin dimethyl ester | 0 | 0·95 |
| Mesoporphyrin | 2 | 0·69 |

* A mixture of monomethyl, and dimethyl esters of protoporphyrin and free protoporphyrin (see the Materials and Methods section).

chloroporphyrin $e_6$, indicating that the electrophilic vinyl groups were hydrated. Since some hydrolysis of ester groups may have occurred during preparation, this porphyrin was re-esterified with diazomethane. Vinylchloroporphyrin $e_6$ was treated in a similar manner. These porphyrins were then divided into two parts, one of which was acetylated (Barrett, 1959). The hydroxylated porphyrins and their acetylated derivatives were then chromatographed with the chloroform–kerosene solvent of Chu *et al.* (1951), in which the difference in $R_F$ of the porphyrin and its acetylated derivative is an indication of the number of hydroxyl groups in the porphyrin (Barrett, 1959). Table 6 shows that the hydroxylated compound 7 behaves as a dihydroxyl compound, and thus confirms the presence of two vinyl groups in the original material.

*Evidence concerning the carboxylic side chains of compound 7.* The number of carboxylic acid groups in compound 7 was estimated by chromatography in lutidine–water. It was found that the porphyrin behaved as a monocarboxylic porphyrin (Table 7). After hydrolysis in 20% hydrochloric acid for 6 hr. it behaved as a dicarboxylic compound. This suggested that compound 7 is a monoester, probably a monomethyl ester, as are earlier compounds in the biosynthesis of chlorophyll (Granick, 1961; Jones, 1963*a*). The presence of the methoxyl group was confirmed by the chromotropic acid method.

## DISCUSSION

The identification of compound 7 as 2,4-divinylphaeoporphyrin $a_5$ monomethyl ester is based on the assumption that, like magnesium protoporphyrin, protochlorophyll, chlorophyll *a* and bacteriochlorophyll, it is a derivative of isomer IX of protoporphyrin, i.e. that the substituents at positions 1, 3, 5 and 8 of the porphyrin nucleus are all $-CH_3$. This assumption is reasonable since the isomer specificity of early enzymic reactions in protoporphyrin biosynthesis appears to preclude the formation of other isomers, and protoporphyrin is believed to be an intermediate in chlorophyll biosynthesis (cf. Granick, 1948). Additional carbonyl, carboxyl, unsaturated or hydroxyl groups should have been detected by the techniques of infrared spectroscopy and chromatography used in this study. The similarity in solubility of the reduced derivative of compound 7 and phaeoporphyrin $a_5$ monomethyl ester suggests that it is unlikely that there is any long-chain alkyl substituent in positions 1, 3, 5 or 8. The suggestion that the carboxyl substituent at position 10 is esterified is based upon analogy with all other chlorophyll compounds of known structure (cf. Smith & Benitez, 1955), and the presence of a methoxyl group strongly suggests that compound 7 has a methyl ester group at position 10.

Under conditions where bacteriochlorophyll biosynthesis is inhibited, and compound 7 accumulates in the cells and medium of *R. spheroides*, a compound spectroscopically identical with phaeophorbide *a* (magnesium-free chlorophyllide *a*, VI in Scheme 1) also accumulates (Jones, 1963*b*). A similar compound has been detected in a mutant of *R. spheroides* (Sistrom, Griffiths & Stanier,

1956). This supports the view that chlorophyllide *a* may be a normal intermediate in bacteriochlorophyll biosynthesis. Since magnesium complexes of chlorins are known to lose their chelated metal ion more readily than magnesium complexes of porphyrins (see, for example, Corwin & Melville, 1955) the accumulation of metal-free pigment in the medium is not unexpected. Scheme 1 for the biosynthesis of bacteriochlorophyll in *R. spheroides* is therefore proposed.

Compounds with formulae (I) (Lascelles, 1956), (III), (IV), (VIII) and magnesium-free (VI) (Jones, 1963*a*, *b*) have all been detected in *R. spheroides*, and (II) is a substrate for enzymic methylation in this organism (Tait & Gibson, 1961) This pathway resembles that proposed for chlorophyll biosynthesis in green plants (cf. Granick & Mauzerall, 1961), but differs in the inclusion of magnesium divinylphaeoporphyrin $a_5$ methyl ester between magnesium protoporphyrin monomethyl ester and magnesium vinylphaeoporphyrin $a_5$ methyl ester. The spectroscopic data of Stanier & Smith (1959) suggest that seed-coat protochlorophyll is very similar to magnesium divinylphaeoporphyrin $a_5$ methyl ester, which thus may be of significance in the biosynthesis of chlorophyll in plants

It now seems possible that magnesium protoporphyrin monomethyl ester is the substrate for the ring-closure reaction that leads to the formation of the isocyclic ring of phaeoporphyrin and that the oxidation of the vinyl side chain to acetyl at position 2, which is necessary for formation of bacteriochlorophyll, occurs after chlorophyllide *a* has been formed. However, the position in the biosynthetic pathway of nearly all the intermediates in chlorophyll biosynthesis that have been detected in mutant or inhibited organisms remains to be conclusively established.

## SUMMARY

1. The protochlorophyll-like material (compound 5) that accumulates when biosynthesis of bacteriochlorophyll in *Rhodopseudomonas spheroides* is inhibited by 8-hydroxyquinoline has been identified as a magnesium phaeoporphyrin. The magnesium is readily removed by acid treatment.

2. The spectroscopic properties of this phaeoporphyrin are consistent with those of 2,4-divinylphaeoporphyrin $a_5$ or possibly of 2-acetylphaeoporphyrin $a_5$.

3. Spectroscopic shifts on treatment with hydroxylamine are consistent with mono-oxime formation, and since a second carbonyl group was not detected by infrared spectroscopy the possibility of an acetyl substituent is eliminated.

4. The presence of two vinyl groups was confirmed by the magnitude of the spectroscopic shift on catalytic hydrogenation; this appears to lead to the formation of phaeoporphyrin $a_5$. Hydroxylation of the vinyl groups of the derived chloroporphyrin gave results consistent with the presence of two hydroxyl groups.

5. Chromatography in lutidine–water showed that the phaeoporphyrin occurs as a monoester which was identified as a monomethyl ester.

6. It is concluded that the bacterial protochlorophyll-like material (compound 5) is magnesium 2,4-divinylphaeoporphyrin $a_5$ monomethyl ester and the role of this compound as an intermediate in plant and bacterial chlorophyll biosynthesis is discussed.

The author thanks Mr J. Barrett and Dr J. E. Falk for useful discussions. He also thanks Fisons Pest Control Ltd., Harston, Cambridge, for financial support.

## REFERENCES

Anderson, A. F. H. & Calvin, M. (1962). *Nature, Lond.*, **194**, 285.
Barrett, J. (1959). *Nature, Lond.*, **183**, 1185.
Chu, T. C., Green, A. A. & Chu, E. J. (1951). *J. biol. Chem.* **190**, 643.
Clezy, P. S. & Barrett, J. (1961). *Biochem. J.* **78**, 798.
Corwin, A. H. & Melville, M. H. (1955). *J. Amer. chem. Soc.* **77**, 2755.
David, D. J. (1960). *Analyst*, **85**, 495.
Falk, J. E. (1961). *J. Chromat.* **5**, 277.
Feigl, F. (1960). *Spot Tests in Organic Analysis*, p. 192. London: Elsevier.
Fischer, H. & Stern, A. (1940). *Die Chemie des Pyrrols*, vol. 2. Leipzig: Akademische Verlagsgesellschaft m.b.H.
Granick, S. (1948). *J. biol. Chem.* **172**, 717.
Granick, S. (1950). *J. biol. Chem.* **183**, 713.
Granick, S. (1961). *J. biol. Chem.* **236**, 1168.
Granick, S. & Mauzerall, D. (1961). In *Metabolic Pathways*, vol. 2, p. 525. Ed. by Greenberg, D. M. New York: Academic Press Inc.
Griffiths, M. (1962). *J. gen. Microbiol.* **27**, 427.
Holt, A. S. & Jacobs, E. E. (1955). *Plant Physiol.* **30**, 553.
Jones, O. T. G. (1963*a*). *Biochem. J.* **86**, 429.
Jones, O. T. G. (1963*b*). *Biochem. J.* **88**, 335.
Koski, V. M. & Smith, J. H. C. (1948). *J. Amer. chem. Soc.* **70**, 3558.
Labbe, R. F. & Nishida, G. (1957). *Biochim. biophys. Acta*, **26**, 437.
Lascelles, J. (1956). *Biochem. J.* **62**, 78.
Lemberg, R. (1953). *Nature, Lond.*, **172**, 619.
Lemberg, R. & Falk, J. E. (1951). *Biochem. J.* **49**, 674.
Lemberg, R. & Legge, J. W. (1949). *Hematin Compounds and Bile Pigments*. London and New York: Interscience Publishers Inc.
Morell, D. B. & Stewart, M. (1956). *Aust. J. exp. Biol. med. Sci.* **34**, 211.
Parker, M. J. (1959). *Biochim. biophys. Acta*, **35**, 496.
Porra, R. J. & Jones, O. T. G. (1963). *Biochem. J.* **87**, 181.
Sistrom, W. R., Griffiths, M. & Stanier, R. Y. (1956). *J. cell. comp. Physiol.* **48**, 459.

Smith, J. H. C. (1960). In *Comparative Biochemistry of Photoreactive Systems*, p. 257. Ed. by Allen, M. B. New York: Academic Press Inc.
Smith, J. H. C. & Benitez, A. (1955). In *Modern Methods of Plant Analysis*, vol. 4, p. 142. Ed. by Paech, K. & Tracey, M. V. Berlin: Springer Verlag.
Stanier, R. Y. & Smith, J. H. C. (1959). *Yearb. Carneg. Instn*, **58**, 336.
Stern, A. & Wenderlein, H. (1935). *Z. phys. Chem.* A, **174**, 81.
Stern, A. & Wenderlein, H. (1936). *Z. phys. Chem.* A, **176**, 81.
Tait, G. H. & Gibson, K. D. (1961). *Biochim. biophys. Acta*, **52**, 614.
Warburg, O. & Gewitz, H.-S. (1951). *Hoppe-Seyl. Z.* **288**, 1.

*Biochem. J.* (1963) **89**, 189

# The Effect of Bile Salts and some Bile-Salt Analogues on the Oxidation of Cholesterol by Liver Mitochondria

BY M. J. LEE AND M. W. WHITEHOUSE
*Department of Biochemistry, University of Oxford*

(*Received* 21 *March* 1963)

Conjugated hydroxycholanic acids (bile salts) are the products of cholesterol catabolism in mammalian liver. Normally, most of the bile-salt output of the liver is returned to the liver after passage through the bile duct and absorption from the small intestine. If this enterohepatic circulation is interrupted by cannulation of the bile duct and removal of the bile, the daily bile-salt production in the rat is considerably increased (Thompson & Vars, 1953; Eriksson, 1957). Bergström & Danielsson (1958) showed that the output of bile salts through a cannula inserted in the upper half (i.e. proximal to the liver) of a bile duct ligated in the middle, was greatly reduced when a solution of bile salt was infused into the small intestine via the lower half of the duct. They therefore concluded that the concentration of bile salts supplied to the liver via the portal blood influences the rate of synthesis of bile salts in the liver. Further evidence for the operation of a 'negative-feedback' mechanism in this system was obtained by Beher & Baker (1958*a*, *b*) and Beher, Baker & Anthony (1959), who showed that feeding of bile acids to rats and mice reduces the rate of mobilization (and also the rate of synthesis) of liver cholesterol.

Conversion of cholesterol into bile acids has not yet been conclusively demonstrated *in vitro*. However, one step in the series of reactions must be the removal of the terminal isopropyl group of the cholesterol side chain. Oxidation of the terminal methyl groups to carbon dioxide by rat-liver slices *in vitro* was observed by Meier, Siperstein & Chaikoff (1952). Subsequently it was shown that this oxidation was carried out by liver mitochondria in the presence of a soluble cofactor ('supernatant factor') (Anfinsen & Horning, 1953; Whitehouse, Staple & Gurin, 1959) and was inhibited by the addition of taurocholate or glycocholate (Whitehouse & Staple, 1959).

Bile salts are surface-active agents and at high concentrations promote mitochondrial lysis. It is therefore questionable whether this inhibition is really a negative-feedback effect, since it could also be the consequence of impaired metabolism in subcellular particles due to the surface-active properties of bile salts. The results of further investigations into the effects on cholesterol oxidation *in vitro* of a range of natural bile salts and some bile-salt analogues are presented in this paper.

A preliminary account of this work has been published (Lee & Whitehouse, 1963).

## EXPERIMENTAL

*Materials.* Sources of supply for special materials were as follows: [26-$^{14}$C]cholesterol, sodium [1-$^{14}$C]octanoate and sodium [2-$^{14}$C]propionate (The Radiochemical Centre, Amersham, Bucks.); Tween 20 (Atlas Powder Co., Wilmington, Delaware, U.S.A.); crystalline bovine serum albumin (C.S.I.R.O., Australia; given by Dr F. J. Hird, University of Melbourne); Asolectin (purified soya phosphatides, Associated Concentrates Inc., Long Island, N.Y., U.S.A.); polyethylene glycol (mol.wt. 1540; L. Light and Co. Ltd., Colnbrook, Bucks.); ATP (disodium salt), AMP, GSH and NAD (Sigma Chemical Co., St Louis, Mo., U.S.A.); Cab-o-Sil (thixotropic gelling agent) (Packard Instruments Ltd., Wembley, Middx.); various cholanic acids (British Drug Houses Ltd., Poole, Dorset; California Corp. for Biochemical Research, Los Angeles, U.S.A.; L. Light and Co. Ltd.; Mann Research Laboratories Inc., New York, U.S.A.; Zori Pharmaceutical and Chemical Industrial Co. Ltd., Tel Aviv, Israel).

*Isolation of liver mitochondria.* Suspensions of mitochondria in aq. 10% (w/v) sucrose were prepared from livers of 3- to 4-month-old white mice (Swiss Hygienic strain), 2- to 3-month-old Wistar rats or 2- to 3-month-old