The Production of Magnesium Protoporphyrin Monomethyl Ester by *Rhodopseudomonas spheroides*

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

(*Received* 20 *August* 1962)

A monoester of magnesium protoporphyrin has been isolated by Granick (1961) from a chlorophyll-less mutant of *Chlorella* and from etiolated barley leaves treated with δ-aminolaevulic acid and αα-bipyridyl. It was considered likely that this material was a methyl ester that Granick then proposed as an intermediate in the biosynthesis of chlorophyll. The extraction from *Rhodopseudomonas spheroides* of an enzyme system which methylates magnesium protoporphyrin to form the monomethyl ester (Tait & Gibson, 1961) has been given as evidence for the participation of this metalloporphyrin in bacteriochlorophyll biosynthesis. In this paper is described the detection and identification of magnesium protoporphyrin monomethyl ester, which was secreted into the medium by intact cells of *R. spheroides*, growing on complete medium and carrying out normal bacteriochlorophyll synthesis.

MATERIALS AND METHODS

Cultures. Rhodopseudomonas spheroides, obtained from Dr J. Orlando, Brandeis University, was grown in the medium of Cohen-Bazire, Sistrom & Stanier (1957), supplemented with 0·1 % of Difco yeast extract, which was found in preliminary experiments to overcome erratic growth.

Incubation conditions. Cultures were grown anaerobically in the light in 500 ml. bottles with ground-glass stoppers. The bottles were placed in a glass-sided water bath at 31° and illuminated by 100 w tungsten lamps so that light falling on the bottles was approximately 150 ft.-candles.

Harvesting of cultures. After 48 hr. growth the cells were centrifuged and washed with 0·85 % NaCl. The cell-free medium was retained. A sample of the washed cells was heated to constant weight at 100° for determination of dry weight. The yield was about 2 g. of dry cells/l. of medium.

Estimation of bacteriochlorophyll. The method of Cohen-Bazire *et al.* (1957) was used and applied to the washed cells.

Chemicals. Mesoporphyrin IX was obtained from Fluka A.G. (Basel). Mesoporphyrin IX dimethyl ester and protoporphyrin IX dimethylester were gifts from Dr J. E. Falk. The ether used was washed with water until free of peroxides. To conform with the usual convention in this field HCl concentrations are expressed as % (w/v).

Spectra. Spectra were determined with a Bausch and Lomb Spectronic 505 recording spectrophotometer.

RESULTS

After incubation the cell suspensions were centrifuged and the supernatant was examined with a hand spectroscope. In addition to the absorption band at 526 mμ, probably due to bacteriophaeophytin (Smith & Benitez, 1955), absorption bands, of approximately equal intensity, were observed at about 550 and 590 mμ. Spectrophotometric examination revealed, in addition, a strong band at 751 mμ, confirming the presence of bacteriophaeophytin. This supernatant was extracted three times with equal volumes of ether, which was concentrated and washed well with water. The spectrum of this crude ether extract is shown in Fig. 1. The strong absorption at 419 mμ and in the

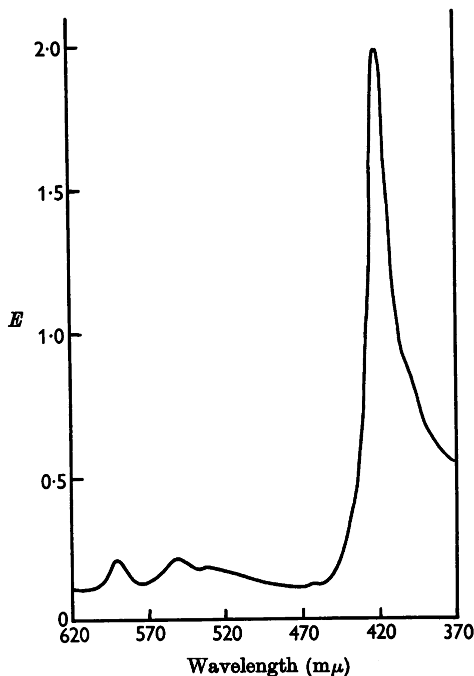

Fig. 1. Absorption spectrum of an ether extract of the cell-free supernatant of *Rhodopseudomonas spheroides* medium, harvested after a 48 hr. growth period. $\lambda_{max.}$: 419, 527, 553 and 591 mμ.

Table 1. *Absorption maxima and band ratios of the magnesium protoporphyrin-like material derived from the medium of* Rhodopseudomonas spheroides *compared with those of magnesium protoporphyrin in ether*

Material from medium	Absorption max. (mμ) ...	419	510	553·5	591
	Band ratio	17·1	0·20	1	1
Magnesium protoporphyrin (Granick, 1961)	Absorption max. (mμ) ...	—	—	553	591
	Band ratio	—	—	1	1
Magnesium protoporphyrin (Granick, 1948)	Absorption max. (mμ) ...	419	510	551	589
	Band ratio	16·9	0·135	1	1

visible region at 553 and 591 mμ resembled that of magnesium protoporphyrin (Granick, 1948, 1961). The ether was extracted with 10% hydrochloric acid; such acid treatment will remove magnesium from magnesium porphyrin complexes (cf. Smith & Benitez, 1955). The aqueous extract was brought to pH 4 with sodium acetate and re-extracted with ether, which was washed with water until free of acid. The spectrum of the ether-soluble material was very similar to that of protoporphyrin (an aetio-type spectrum, having absorption bands in ether at 404, 503, 536, 576, 605 and 633 mμ), but it was found that the material was less easily extracted from ether into 2·5% hydrochloric acid than was protoporphyrin. This decrease in acid solubility would be consistent with esterification of one or both of the propionic acid side chains of a porphyrin like protoporphyrin (cf. Falk, 1961). From the observations of Granick (1961) it seemed possible that a major component of the ether extract of the medium was magnesium protoporphyrin monomethyl ester. This was confirmed by the purification of the material followed by a study of its properties, as described below.

Column chromatography of the crude ether extract. A sample of the ether extract was washed with 0·02N-ammonia–methanol (4:1, v/v) to decrease the amount of bacteriophaeophytin-like material and extracted into the 0·1N-ammonia–methanol (3:1, v/v) solvent of Granick (1961). The alkaline extract was neutralized with sodium dihydrogen phosphate and extracted into fresh ether, and the ether solution was washed with water, concentrated and dried over anhydrous sodium sulphate. This material was placed on a powdered sucrose column, washed with light petroleum (b.p. 60–80°) and the chromatogram developed with ether–light petroleum (b.p. 60–80°) (3:2, v/v). Three bands developed and the fastest-moving, red fluorescent band (band I) was eluted and collected. Bands II and III were not further examined. Some properties of the absorption spectrum of the eluted material are given in Table 1 and compared with those of magnesium protoporphyrin (Granick, 1948, 1961).

Effect of brief acid treatment. A sample of the band I material was extracted from the ether–light petroleum eluting solvent into 10% hydrochloric acid, brought to pH 4 with sodium acetate, re-extracted into ether and washed with water until free of acid. Shaking with the acid for several minutes was necessary before all the fluorescent band I material was extracted from the ether. The product of this treatment had a spectrum in ether identical with that of protoporphyrin and the maximum in the Soret region in 2·5% hydrochloric acid (408 mμ) also corresponded to that of protoporphyrin.

Table 2. *R_F values of some porphyrins chromatographed in* 2,6-*lutidine–water* (13:7, v/v.)

The chromatography was carried out by a modification (see the text) of the method of Eriksen (1953).

Porphyrin	No. of free carboxyl groups	R_F
Protoporphyrin dimethyl ester	0	0·95
Protoporphyrin dimethyl ester, partly hydrolysed*	0, 1 and 2	0·94; 0·87; 0·69
Protoporphyrin dimethyl ester, completely hydrolysed	2	0·71
Mesoporphyrin	2	0·72
Mesoporphyrin dimethyl ester	0	0·94
Porphyrin from medium	—	0·88
Porphyrin from medium, completely hydrolysed	—	0·71

* The ester was partly hydrolysed by treatment with 10% HCl for 3 hr.

Hydrolysis and chromatography of the acid-treated band I material. A sample of the porphyrin was dissolved in 20% hydrochloric acid, and left for 10 hr. in the dark at 4°. This prolonged acid treatment would hydrolyse any ester groups which may be present in the porphyrin (Falk, 1961). The porphyrins before and after hydrolysis were chromatographed in the 2,6-lutidine–water (13:7, v/v) solvent of Eriksen (1953), modified to run as a descending system, in which the R_F of a porphyrin is an indication of the number of free carboxyl groups on the side chains. The R_F values obtained (Table 2) indicate that the porphyrin has one free carboxyl group before hydrolysis and two after hydrolysis, suggesting that one carboxyl group is esterified in the original material.

Test for a methoxyl group. It seemed possible

that the porphyrin obtained by the process of acid extraction of the original ether-soluble material was a monomethyl ester. A sample of the porphyrin was heated to 120° with benzoyl peroxide and the formation of formaldehyde detected with the chromotropic acid colour reagent as described by Feigl (1960). This indicated the presence of methoxyl groups or *N*-methyl groups in the original material.

Formation of the pyridine haemochromogen on the briefly acid-treated band I material. Iron was introduced into this protoporphyrin-like material by the method of Morell, Barrett & Clezy (1961). The resulting haem was dissolved in the alkaline pyridine reagent of Paul, Theorell & Åkeson (1953) and the spectrum of the reduced pyridine haemochromogen recorded after the addition of sodium dithionite. This spectrum (maxima at 557 and 526 mμ) was identical with that of pyridine protohaemochromogen. No haem could have been formed if a *N*-methyl group were present in the parent porphyrin, which must, therefore, have possessed a methoxyl group.

These tests confirm that the band I material was magnesium protoporphyrin monomethyl ester.

Effect of iron deficiency during growth of Rhodopseudomonas spheroides. Lascelles (1956) showed that *R. spheroides* grown in iron-deficient media produces free porphyrins, principally coproporphyrin, which accumulate in the medium. The extraction technique used by Lascelles would not have detected a compound of the magnesium protoporphyrin type since acid was added at an early stage. *R. spheroides* was therefore grown in media of various iron concentrations and the cell-free media were extracted with ether. Spectroscopic examination showed that these extracts contained magnesium protoporphyrin-like material and bacteriophaeophytin in various amounts. Extracts of iron-deficient media contained no detectable bacteriophaeophytin. The ether extracts were extracted with 8% hydrochloric acid, resulting in conversion of the magnesium complex into a protoporphyrin-like material which was determined spectrophotometrically as protoporphyrin in 2·5% hydrochloric acid (ϵ at 408 mμ $2{\cdot}62\times10^5$; Rimington, 1960). The results given in Fig. 2 show that in iron-deficient cultures there was an appreciable increase in magnesium protoporphyrin-like material. No measures were taken to exclude traces of iron, which may be present as impurities in the 'iron-free' media. It was confirmed by paper chromatography that the material produced in iron deficiency possessed only one free carboxyl group. No free magnesium protoporphyrin-like material was detected by spectroscopic examination of whole cells, or in acetone–methanol (7:2, v/v) extracts of the cells.

DISCUSSION

Granick (1961) has detected protoporphyrin monomethyl ester produced by mutants of *Chlorella* together with small amounts of a magnesium protoporphyrin monoester. A similar complex has now been isolated from the medium of *R. spheroides* and identified as the monomethyl ester. This is the first report of the production of such a compound by normal cultures of intact wild-type cells and supports the postulate of Granick that this material is a normal metabolite. The work with mutants of *Chlorella* has led to a scheme of biosynthesis of bacteriochlorophyll by the following pathway (cf. Gibson, Matthew, Neuberger & Tait, 1961): protoporphyrin → magnesium protoporphyrin or protoporphyrin monomethyl ester → magnesium protoporphyrin monomethyl ester → → → magnesium vinyl phaeoporphyrin a_5 → magnesium vinyl phaeoporphyrin a_5 phytyl ester → chlorophyll *a* → bacteriochlorophyll.

Such a scheme is attractive since it presents a common route to chlorophyll *a* in green plants and bacteria, but work by Griffiths (1962) shows that two mutants of *R. spheroides* that are unable to synthesize bacteriochlorophyll produce compounds at the levels of oxidation of magnesium vinyl phaeoporphyrin a_5 and chlorophyll *a* respectively but which differ from them in spectroscopic properties and, presumably, substituents. This suggests that the pathways of biosynthesis of chlorophyll *a* and bacteriochlorophyll diverge at an early stage. The detection of the production of

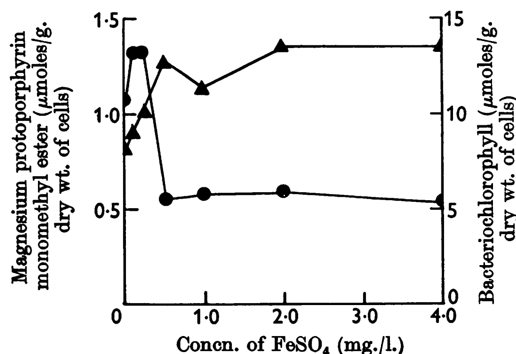

Fig. 2. Effect of low-iron media on the production of magnesium protoporphyrin monomethyl ester (●) and bacteriochlorophyll (▲) by *Rhodopseudomonas spheroides*. Magnesium protoporphyrin monomethyl ester was determined in ether extracts of the cell-free growth medium after its conversion by mild acid treatment into protoporphyrin monomethyl ester and measurement of the $E^{408}_{1\,\mathrm{cm.}}$ in 2·5% HCl. Bacteriochlorophyll was determined in acetone–methanol (7:2, v/v) extracts of cells.

magnesium protoporphyrin monomethyl ester by intact wild-type cells of *R. spheroides* indicates that the divergence takes place at a stage between this compound and magnesium vinyl phaeoporphyrin a_5. The detection by Tait & Gibson (1961) of an enzyme system in *R. spheroides* that methylates magnesium protoporphyrin supports this view, although these authors do not report the specificity of this enzyme with regard to side chains on the porphyrin nucleus.

In low-iron media the synthesis of bacteriochlorophyll was decreased, but there was increased production of magnesium protoporphyrin monoester (Fig. 2). Although compounds of the magnesium vinyl phaeoporphyrin a_5 and phaeophytin a type would be extracted into ether under the conditions used (O. T. G. Jones, unpublished work), none was detected and bacteriophaeophytin was absent from the iron-deficient media. This suggests that iron may be required for the enzymic transformations of the side chains of magnesium protoporphyrin monoester in addition to its activity during the early stages of porphyrin biosynthesis, where it has been shown that δ-aminolaevulate synthase is inhibited by Fe^{2+} ions (Brown, 1958; Burnham, 1962).

SUMMARY

1. An ether-soluble pigment secreted into the medium by intact cells of *Rhodopseudomonas spheroides* has been purified by chromatography on a powdered sucrose column.

2. The spectroscopic and chromatographic properties of this purified material and its derivatives produced by acid treatment were studied and the material was identified as magnesium protoporphyrin monomethyl ester.

3. The ester was not detected in whole cells or in acetone–methanol extracts of whole cells.

4. In low-iron media there was increased secretion of this ester into the medium and bacteriochlorophyll production decreased.

REFERENCES

Brown, E. G. (1958). *Nature, Lond.*, **182**, 313.
Burnham, B. F. (1962). *Biochem. biophys. Res. Commun.* **7**, 351.
Cohen-Bazire, G., Sistrom, W. R. & Stanier, R. Y. (1957). *J. cell. comp. Physiol.* **49**, 25.
Eriksen, L. (1953). *Scand. J. clin. Lab. Invest.* **5**, 155.
Falk, J. E. (1961). *J. Chromat.* **5**, 277.
Feigl, F. (1960). *Spot Tests in Organic Analysis*, p. 192. London: Elsevier.
Gibson, K. D., Matthew, M., Neuberger, A. & Tait, G. H. (1961). *Nature, Lond.*, **192**, 204.
Granick, S. (1948). *J. biol. Chem.* **175**, 333.
Granick, S. (1961). *J. biol. Chem.* **236**, 1168.
Griffiths, M. (1962). *J. gen. Microbiol.* **27**, 427.
Lascelles, J. (1956). *Biochem. J.* **62**, 78.
Morell, D. B., Barrett, J. & Clezy, P. S. (1961). *Biochem. J.* **78**, 793.
Paul, K.-G., Theorell, H. & Åkeson, Å. (1953). *Acta chem. scand.* **7**, 1284.
Rimington, C. (1960). *Biochem. J.* **75**, 620.
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.
Tait, G. H. & Gibson, K. D. (1961). *Biochim. biophys. Acta*, **52**, 614.

Biochem. J. (1963) **86**, 432

Substrate Competition in the Respiration of Animal Tissues

THE METABOLIC INTERACTIONS OF PYRUVATE AND α-OXOGLUTARATE IN RAT-LIVER HOMOGENATES

By R. J. HASLAM* and H. A. KREBS
Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, University of Oxford

(*Received* 23 *July* 1962)

A readily oxidizable substrate—an intermediate or a starting material—often inhibits the oxidation of other substrates when added to respiring material (Krebs, 1935; Edson, 1936). In terms of enzyme chemistry this means that oxidizable substrates and intermediates derived from them compete with each other for the joint pathway of electron transport to molecular oxygen or for a shared co-factor. The present investigation is concerned with the detailed study of the competitive and other interactions of pyruvate, α-oxoglutarate and endogenous substrates in respiring rat-liver homogenates.

* Present address: Sir William Dunn School of Pathology, University of Oxford.