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MAGNESIUM 2,4-DIVINYLPHAEOPORPHYRIN a_5 MONOMETHYL ESTER, A PROTOCHLOROPHYLL-LIKE PIGMENT PRESENT IN SOME UNICELLULAR FLAGELLATES

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Abstract—The isolation of a protochlorophyll-like pigment from some unicellular flagellates, having the spectrophotometric, i.r. spectroscopic and solubility properties of magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester, is described. It occurs in amounts ranging from 2 to 9 per cent of total chlorophyll pigment in the marine flagellates: *Micromonas pusilla*; *Micromonas squamata* (Plymouth Nos. 281A, 289, 290); *Heteromastix longifilis*; *Heteromastix* sp. (Plymouth No. 198) and *Pyramimonas amylifera*. It was not detected in the marine flagellate *Heteromastix rotunda*, or in the freshwater flagellates *Monomastix caeca*; *Spermatozopsis*; *Pedinomonas minor* or *Pedinomonas tuberculata*. The relationship of the pigment to the classification of these flagellates and to the biosynthesis of chlorophyll *a* is discussed.

INTRODUCTION

DURING investigations into the chlorophyll *c* status of some pigmented flagellates, using the method of Parsons,[1] a light green pigment having an absorption spectrum similar to that of protochlorophyll was detected. The present paper reports on the isolation and identification of this pigment and gives some notes on its distribution in unicellular flagellates.

RESULTS

Figure 1 shows the absorption spectra of the pigment from *Micromonas squamata* (Plymouth 289) in 90 per cent acetone, isolated by the method of Parsons,[1] before and after hydrochloric acid treatment. The absorption maxima of the pigment are 630, 575–80 and 441 mμ; and of the phaeophytin 572 and 423 mμ. Paper chromatography of extracts of this type indicated that there was trace contamination with carotenoids, as is also indicated by the asymmetry at about 470 mμ in Fig. 1. This probably obscures smaller absorption maxima.

The absorption spectrum of the pigment isolated by the method of Granick[2] from *Micromonas squamata* (Plymouth 290) is shown in Fig. 2. The pigment is not absolutely pure, as is shown by its slight absorption at 660 mμ. The absorption maxima and relative intensities are shown in Table 1 and compared with those of pigments 5 and 7 obtained by Jones[3] from *Rhodopseudomonas spheroides*, and with protochlorophyll.[4]

Infrared spectra of the phaeophytin of the protochlorophyll-like pigment (which showed no absorption at 665 mμ) in carbon tetrachloride solution showed peaks at about 1713 cm^{-1} and 1738 cm^{-1}. The former showed a very small shoulder at a slightly lower frequency. Holt and Jacobs[5] have shown that chlorophyll pigments and their derivatives show peaks

[1] T. R. PARSONS, *J. Marine Research* **21**, 164 (1963).
[2] S. GRANICK, *J. Biol. Chem.* **183**, 713 (1950).
[3] O. T. G. JONES, *Biochem. J.* **88**, 335 (1963).
[4] V. M. KOSKI and J. H. C. SMITH, *J. Am. Chem. Soc.* **70**, 3558 (1948).
[5] A. S. HOLT and E. E. JACOBS, *Plant Physiol.* **30**, 553 (1955).

at 1680–1710 cm^{-1}, due to the isocyclic-ring carbonyl group: at 1702–1725 cm^{-1} due to the carbonyl group of the carboxyl C_7 and at 1730–1740 cm^{-1} due to the ester carbonyl group. The present results tend to confirm the presence of the isocyclic ring and the ester grouping. The results are similar (but about 10 cm^{-1} higher in each case) to those obtained by Jones[6] in unspecified solvent. The absence of a peak in the 1660 cm^{-1} region indicates that no acetyl group is present.

If the spectrum was determined using the pigment in the solid state the two peaks were shifted about 14 cm^{-1} to higher frequencies.

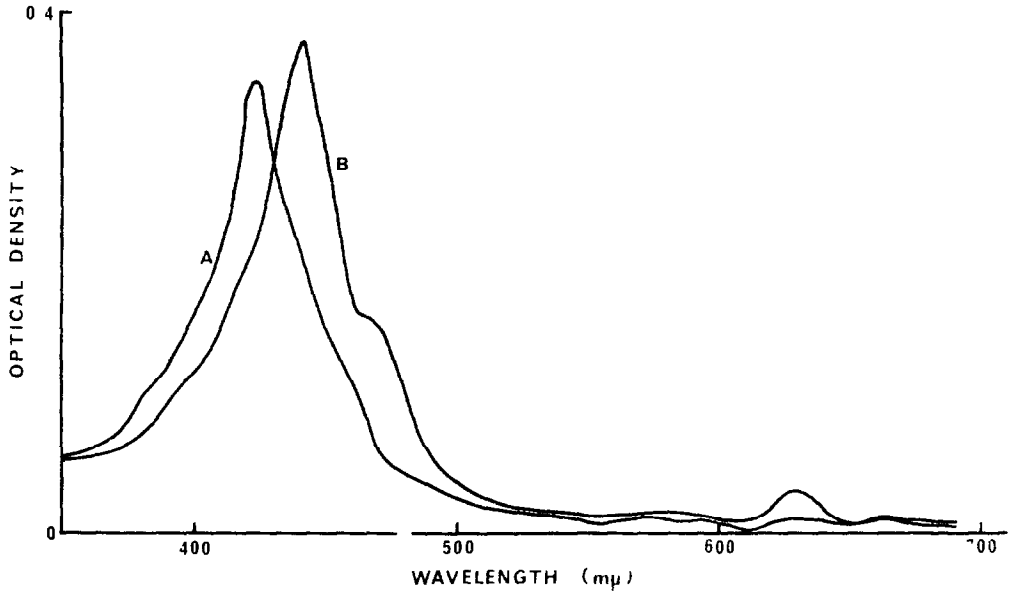

FIG. 1. THE ABSORPTION SPECTRA OF THE PROTOCHLOROPHYLL-LIKE PIGMENT IN 90% AQUEOUS ACETONE SOLUTION, BEFORE AND AFTER HYDROCHLORIC ACID TREATMENT, ISOLATED BY THE METHOD OF PARSONS[1] FROM *Micromonas squamata* (PLYMOUTH 289).

Broken line = protochlorophyll-like pigment; continuous line = pigment after hydrochloric acid treatment.

Additionally peaks were obtained at 2857 cm^{-1} and 2933 cm^{-1} (in both the solid state and carbon tetrachloride solution) which had a greater magnitude than the 1713 cm^{-1} and 1738 cm^{-1} peaks. A phytol group causes adsorption at 2916–2930 cm^{-1} but its magnitude is less than the 1680–1710 cm^{-1} and 1730–1740 cm^{-1} peaks. The absorption obtained is therefore attributed to unidentified hydrocarbon impurities in the sample. The solubility of the pigment phaeophytin in 15 per cent hydrochloric acid (w/v) also indicates that it is not a phytyl ester. Results for the other organisms examined for the presence of the pigment are shown in Table 2.

[6] O. T. G. JONES, *Biochem. J.* **89**, 182 (1963).

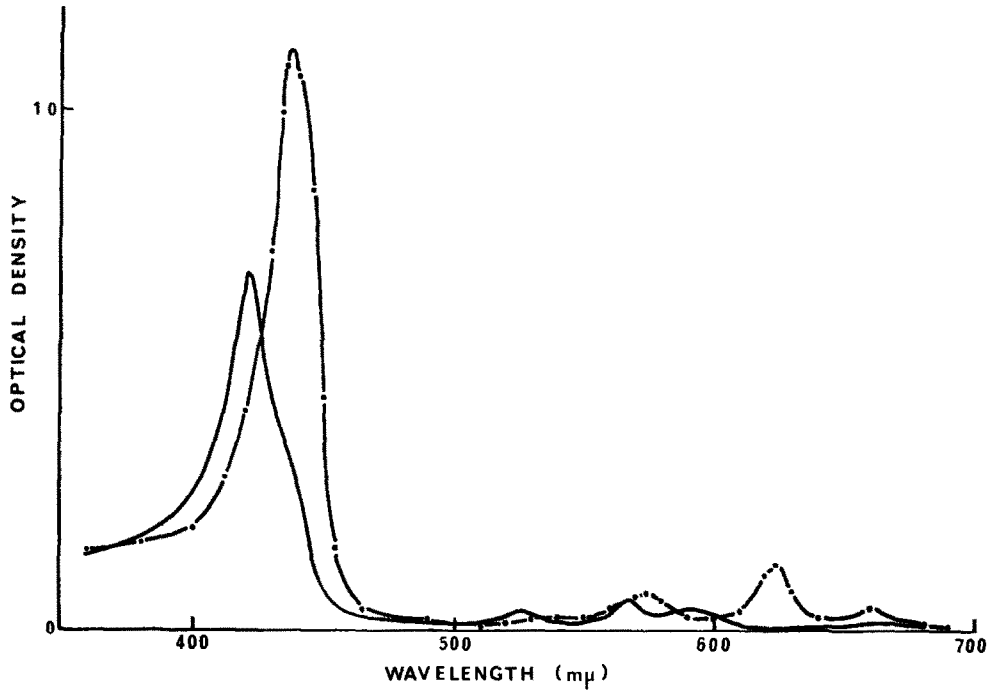

FIG. 2. THE ABSORPTION SPECTRA OF THE PROTOCHLOROPHYLL-LIKE PIGMENT IN ETHEREAL SOLUTION, BEFORE AND AFTER HYDROCHLORIC ACID TREATMENT, ISOLATED BY THE METHOD OF GRANICK[2] FROM *Micromonas squamata* (PLYMOUTH 290).

B = protochlorophyll-like pigment; *A* = pigment after hydrochloric acid treatment. Note that curves *A* and *B* are not at the same pigment concentrations.

TABLE 1. THE ABSORPTION MAXIMA AND BAND RATIOS IN ETHER OF THE PIGMENT OF *Micromonas squamata* (PLYMOUTH 290) AND ITS PHAEOPHYTIN COMPARED WITH THOSE OF OTHER COMPOUNDS

Compound	Spectral properties λ_{max}(mμ) and ratios (%) in brackets				
Pigment	437·5	537	574	624	
	(100)	(2·1)	(5·9)	(11·1)	
Compound 5†	438	537	574	624	
	(100)	(2·8)	(6·8)	(12·2)	
Protochlorophyll	432	535	571	623	
	(100)	(2·2)	(4·6)	(12·1)	
Pigment phaeophytin	421	527	567–8	590	
	(100)	(5·4)	(8·6)	(5·4)	
Pigment phaeophytin‡	421	527	568	590	642
	(100)	(6·3)	(9·4)	(6·5)	(0·97)
Compound 7*†	421·5	527	568	590	642
	(100)	(5·4)	(9·1)	(6·1)	(0·93)
Protophaeophytin	417	524	565	585	638
	(100)	(4·57)	(9·4)	(6·25)	(0·98)

* Obtained from compound 5 by HCl treatment.
† From *Rhodopseudomonas spheroides*.[3]
‡ From *Micromonas pusilla*.

TABLE 2. THE DISTRIBUTION OF MAGNESIUM 2,4-DIVINYLPHAEOPORPHYRIN a_5 MONOMETHYL ESTER IN VARIOUS UNICELLULAR ALGAE AND THE VARIOUS METHODS OF INVESTIGATION USED

Alga	Strain	Method* Parsons[1]	Jeffrey[16]	Granick[2]	Strain *et al.*[17]
Micromonas pusilla	Plym. 27	+	+	+	
Micromonas squamata	Plym. 281A	+			
	Plym. 289	+	+		−
	Plym. 290	+	+	+	−
Heteromastix rotunda	Plym. 210	−			
Heteromastix longifilis	Plym. 58	+	+	−	
Heteromastix sp.	Plym. 198	+	+	−	
Pyramimonas amylifera	Plym. 246	+	+		
Monomastix		−			
Spermatozopsis		−			
Pedinomonas minor	17B	−	−		
Pedinomonas tuberculata	1965/2	−			

* + indicates the presence of the pigment: − indicates that it was not detected.

The total percentage chlorophyll pigment composition of the flagellates is shown in Table 3. The chlorophyll *a* and *b* concentrations were determined in 90 per cent acetone extracts using the specific absorption coefficients of Parsons and Strickland.[7] No correction has been made for any absorption at 645 mμ or 665 mμ due to magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester. The approximate concentration of the protochlorophyll-like pigment was determined by the optical density of hydrochloric acid-treated acetone extracts prepared by the method of Parsons.[1] The assumption was made that the ϵ_{mM} at the Soret maximum was 193, equal to that of vinylphaeoporphyrin a_5[2], in calculating the results. It can be seen that the results generally fall within the range 3–6 per cent of total chlorophyll pigment. Isolation of the protochlorophyll-like pigment by other methods also suggests that *Heteromastix longifilis* has a lower concentration than the other algae.

TABLE 3. THE CHLOROPHYLL PIGMENT COMPOSITION OF SOME ALGAE

Algae	Plymouth number	% Total chlorophyll pigment Chlorophyll *a*	Chlorophyll *b*	Protochlorophyll-like pigment
Micromonas pusilla	27	54·3	42·2	3·5
Micromonas squamata	281A	62·8	32·9	4·3
Micromonas squamata	289	66·4	28·3	5·3
Micromonas squamata	289	60·9	29·7	9·4
Micromonas squamata	290	61·9	31·7	6·4
Micromonas squamata	290	66·7	28·9	4·4
Heteromastix sp.	198	59·5	37·3	3·2
Heteromastix longifilis	58	58·6	39·4	2·0
Pyramimonas amylifera	246	63·5	33·9	2·6

[7] T. R. PARSONS and J. D. H. STRICKLAND, *J Marine Research* **21**, 155 (1963).

As regards the possible derivation of the pigment from the bacteria in the algal cultures rather than from the algae themselves, it can be seen that a number of bacteria-containing cultures display no pigment. Also, no bacteriochlorophyll was detected on chromatography of any of the pigment extracts. At this point it should perhaps be mentioned that on column chromatography of pigment extracts of *Micromonas pusilla* (Plymouth 290) using icing sugar the pigment was strongly adsorbed and was the next to the last pigment to be eluted (using light petrol containing 2 per cent *n*-propanol), the last pigment to be eluted being neoxanthin. Column or paper chromatography of all the organisms containing the protochlorophyll-like pigment showed a number of red or orange-red xanthophylls, which are the major xanthophyll components in these organisms. These are at present under investigation. Preliminary results suggest that these pigments contain two or more free-hydroxy or keto groups.

DISCUSSION

The spectrophotometric, i.r. spectroscopic and solubility properties of the protochlorophyll-like pigment and of its phaeophytin are consistent with their being identical with the compounds 5 and 7 respectively of Jones,[3] which were isolated from *Rhodopseudomonas spheroides* grown on a medium containing 8-hydroxyquinoline. Stanier and Smith[8] and Griffiths[9] have also described mutants of *Rhodopseudomonas spheroides* that were unable to synthesize bacteriochlorophyll and accumulated a compound which was called bacterial protochlorophyll[8] and which had similar properties to the compound 5 of Jones.[3] Compound 5 was later identified by Jones[6] as magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester. It has been suggested that this pigment is an intermediate both in the biosynthesis of chlorophyll *a* in green plants and of bacteriochlorophyll in photosynthetic bacteria.[6] Its suggested position on the biosynthetic pathway lies between magnesium protoporphyrin monomethyl ester and magnesium vinylphaeoporphyrin a_5 monomethyl ester.[3] More recently Jones,[10] in a preliminary communication, has reported that in addition to protochlorophyll a pigment is present in the seed-coats of Cucurbitaceae which closely resembles the protochlorophyll-like pigment. It is now obvious that the name bacterial protochlorophyll[8] should be abandoned as the pigment is not confined solely to bacteria.

The accumulation of the pigment in the algae described ind cates that either it is being formed by a branch reaction from the main biosynthetic pathway to chlorophyll *a*, or that the enzymes concerned with its conversion to chlorophyll *a* are lower in concentration relative to other organisms. These algae may, therefore, be useful for the determination of the biosynthetic pathways to chlorophyll.

It was hoped that the distribution of the pigment among algal genera might be of taxonomic value and this possibility still exists, though the results are not yet sufficient to be used with confidence for such a purpose. The presence of the pigment in both species attributed to *Micromonas* but in neither species of *Pedinomonas* is in accord with the classification based on fine structure[11, 12] but this agreement is somewhat offset by the lack of uniformity among the three species of *Heteromastix*. Surveying Table 2 as a whole, the line of separation between *Pyramimonas* (with the pigment) and *Monomastix* (without) may perhaps coincide

[8] R. Y. Stanier and J. H. C. Smith, *Yearbook. Carneg. Instn.* **58**, 336 (1959).
[9] M. Griffiths, *J. Gen. Microbiol.* **27**, 427 (1962).
[10] O. T. G. Jones, *Biochem. J.* **96**, 6P (1965).
[11] I. Manton and M. Parke, *J. Marine Biol. Assoc. United Kingdom* **39**, 275 (1960).
[12] H. Ettl and I. Manton, *Nova Hedwigia* **8**, 421 (1964).

with the boundary between Loxophyceae and Prasinophyceae[13] but much further work will be needed before a phyletic decision of this kind will be possible.

EXPERIMENTAL

Cultures

The following marine organisms were kindly provided by Dr. Mary Parke, of the Plymouth Laboratory: *Micromonas pusilla* (Plymouth No. 27); *Micromonas squamata* (various strains, Plymouth Nos. 281A, 289 and 290); *Heteromastix rotunda* (Plymouth No. 210); *Heteromastix longifilis* (Plymouth No. 58); *Heteromastix* sp.* (Plymouth No. 198); *Pyramimonas amylifera* (Plymouth No. 246). These were maintained in Erdschrieber culture media.

The following freshwater organisms were available in culture in the Department: *Monomastix caeca* and *Spermatozopsis*, isolated and originally supplied by Dr. J. H. Belcher of the Freshwater Biological Association's Windermere laboratory; *Pedinomonas minor* (strain 17b) isolated and originally supplied by Dr. H. Ettl, Czechoslovakia; and *Pedinomonas tuberculata* (No. 1965/2) supplied from the Culture Collection of Algae and Protozoa, Cambridge. These organisms were grown in the medium described in Ricketts[14] for *Pedinomonas minor*.

All these organisms belong to the Chlorophyta *sens. lat.* though they are not members of the Chlorophyceae *sens. strict.* (Cf. Christensen[13] by whom they are relegated to two separate groups under the names of Prasinophyceae and Loxophyceae.) For further comment on the classification see p. 227.

All cultures were unialgal† but not bacteria-free (except that of *Pedinomonas minor* which had been rendered axenic by a dilution technique) and were grown at 14° with 16 hr illumination (150 lm ft^{-2}) and 8 hr of darkness per day.

Harvesting

Cultures were generally harvested, after 2–4 weeks of growth, by centrifugation in 250 ml volumes at 1000 *g* for 10 min, the supernatant then being decanted. The deposits were then resuspended in a little supernatant with the aid of a pipette and quantitatively transferred to a conical centrifuge tube, which was then centrifuged at 1000 *g* for about 10 min. The supernatant was then decanted and the deposit immediately resuspended in 90 per cent aqueous acetone in the dark. The pigments were extracted almost immediately and the tube centrifuged and supernatant decanted.

Pigments

All the organisms listed contain chlorophylls *a* and *b*, but not *c*, together with carotenoids. In addition, the protochlorophyll-like pigment (hereafter called the pigment), when present, could be extracted by the following methods:

(1) By using the technique described by Parsons[1] for the determination of chlorophyll *c*. This method was subsequently used routinely as a screening method for initial determination of the presence or absence of the pigment.

* This undescribed species was formerly listed as *Bipedinomonas* sp. a name which has recently been suppressed as a synonym of *Heteromastix* as discussed by Manton *et al.*[15]

† The *Pyramimonas* culture was supplied contaminated with a small colourless flagellate. The culture examined contained approximately 5 per cent of these cells, of total cell volume not more than 1 per cent of that of *Pyramimonas*.

[13] T. CHRISTENSEN, *Botanik*, Bind II, *Systematisk Botanik*, Nr. 2, Alger. Munksgaard, Copenhagen (1962).
[14] T. R. RICKETTS, *Phytochem.* **4**, 725 (1965).
[15] I. MANTON, D. G. RAYNS, H. ETTL and M. PARKE, *J. Marine Biol. Assoc. United Kingdom* **45**, 241 (1965).

(2) Additionally the paper-chromatographic separation of pigments using the method of Jeffrey[16] was employed. The pigment was found to have an R_f which was very similar to that described for chlorophyll *c* and to be well separated from the other pigments. The pigment spot was cut out and eluted with ether for spectrophotometric examination.

(3) The pigment was also extracted by the method described by Granick[2] for magnesium vinyl phaeoporphyrin a_5, involving petroleum ether extraction of an acetone extract of the pigments to remove chlorophyll, carotenoids and fatty substances; and then extraction of the pigment from the acetone extract with ether, after first diluting with water and acidifying with M/15 KH_2PO_4. In contrast to the pigment of Granick[2] the pale green pigment was not extracted with 0·05 M ammonia buffer, pH 9·5.

(4) The pigment could also be isolated by column chromatography on icing sugar (British Sugar Corporation) using the method of Strain *et al.*[17] The pigment was strongly adsorbed, but, in contrast to chlorophyll *c*, could be eluted with petroleum ether containing 2 per cent *n*-propanol. Absorption curves were determined using a Unicam SP600 spectrophotometer.

Preparation of the Phaeophytin

The 90 per cent acetone extracts were treated with concentrated HCl as described by Parsons.[1] The phaeophytin pigment was stable for at least 90 min under these conditions. Ethereal solutions were shaken with a small volume of concentrated HCl (~ 0·02 ml/10 ml) and later washed with water to remove the acid. For i.r. spectroscopy ethereal solutions of the pigment (obtained from about 10 l of culture) were shaken with 5 per cent HCl (w/v) until complete conversion to the phaeophytin had occurred and then the ethereal layer washed with water to remove the acid. Infrared spectra were determined using a Grubb Parsons Double Beam Grating Recording Spectrophotometer, Type GS2.

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[16] S. W. Jeffrey, *Biochem. J.* **80**, 336 (1961).
[17] H. H. Strain, W. M. Manning and G. Hardin, *Biol. Bull.* **86**, 169 (1944).