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THE PIGMENTS OF THE PHYTOFLAGELLATES, *PEDINOMONAS MINOR* AND *PEDINOMONAS TUBERCULATA*

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Abstract—The average cell pigment content of the phytoflagellates, *Pedinomonas minor* and *P. tuberculata* has been investigated. *P. minor* contained α-, β- and γ-carotene, an unidentified carotene, lutein, zeaxanthin, lutein 5,6-epoxide, violaxanthin, luteoxanthin, neoxanthin, neochrome, chlorophylls *a* and *b* in the following amounts: 4·1, 18·0, 18·4, 2·6, 27·8, 0·9, 2·1, 1·8, 1·5, 5·4, 0·5, 285·7 and 96·4 pg per average cell (× 1000) respectively. *P. tuberculata* contained α-, β- and γ-carotene, two unidentified carotenes, lycopene, lutein, zeaxanthin, violaxanthin, luteoxanthin, neoxanthin, neochrome, chlorophylls *a* and *b* in the following amounts: 0·4, 6·7, 2·8, 0·5, 0·4, 1·3, 15·4, 1·2, 9·0, 1·8, 5·6, 1·3, 170·1 and 79·7 pg per average cell (× 1000) respectively. Both flagellates are distinct from other green flagellates so far investigated in their high γ-carotene levels. *P. minor* also differs in having lutein 5,6-epoxide, and *P. tuberculata* in having lycopene. The organisms are quite distinct in their carotenoid composition from *Micromonas*. The approximate molecular concentration of chlorophylls and carotenoids per unit area of plastid unit membrane have been calculated to be $4{\cdot}8 \times 10^5$ and $1{\cdot}8 \times 10^5$ molecules per μ^2 for *P. minor* and $4{\cdot}2 \times 10^5$ and $1{\cdot}3 \times 10^5$ molecules per μ^2 for *P. tuberculata*. These values are of the same order as those obtained by other workers for a wide variety of photosynthetic types.

INTRODUCTION

THE rank and taxonomic position of *Pedinomonas* is uncertain. The *Pedinomonas* group consists of green unicellular flagellates containing both chlorophyll *a* and chlorophyll *b*.[1,2] The light and electron microscopic cell structure of *P. tuberculata* and of *Micromonas* were described and the phyletic positions discussed in Manton and Parke[3] who concluded that it was premature to detail the status of the organisms with the information available. A more recent cytological and fine structural study of *P. minor*[4] (type species of the genus) reached the same conclusion, but the distinctness of the genus from Volvocales was stressed. The chemical composition of *P. minor* has already been described.[5] The present work was undertaken in the hope of clarifying the taxonomic position of *Pedinomonas*.

RESULTS

The carotenoid composition of *P. minor* and *P. tuberculata* is shown in Table 1. The absorption maxima of the pigments in various solvents are shown in Table 2, which also shows the maxima after hydrochloric acid treatment of the 5,6-epoxide xanthophylls. All the xanthophylls were treated in this way.

[1] R. HARDER and W. KOCH, *Arch. Mikrobiol.* **21**, 1 (1954).
[2] T. R. RICKETTS, *Phytochem.* **4**, 725 (1965).
[3] I. MANTON and M. PARKE, *J. Marine Biol. Ass. U.K.* **39**, 275 (1960).
[4] H. ETTL and I. MANTON, *Nova Hedwigia* **8**, 421 (1964).
[5] T. R. RICKETTS, *Phytochem.* **5**, 67 (1966).

TABLE 1. THE CHLOROPHYLL AND CAROTENOID COMPOSITION OF *Pedinomonas minor* AND *Pedinomonas tuberculata*

(Carotenoids in order of increasing adsorption in each case.)

Pigment	*P. minor*		*P. tuberculata*	
	pg/average cell (× 1000)	% total carotenoid	pg/average cell (× 1000)	% total carotenoid
α-Carotene	4·07	4·9	0·40	0·9
β-Carotene	17·95	21·7	6·71	14·5
Unidentified 1	2·56	3·1	—	—
Unidentified 2	—	—	0·47	1·0
γ-Carotene	18·40	22·2	2·78	6·0
Unidentified 3	—	—	0·43	0·9
Lycopene	—	—	1·32	2·9
Lutein	27·76	33·5	15·37	33·1
Zeaxanthin	0·90	1·1	1·20	2·6
Lutein 5,6-epoxide	2·10	2·5	—	—
Violaxanthin	1·75	2·1	9·01	19·4
Luteoxanthin	1·49	1·8	1·77	3·8
Neoxanthin	5·36	6·5	5·60	12·1
Neochrome	0·47	0·6	1·31	2·8
Total carotenoids	82·81	100·0	46·37	100·0
Chlorophyll *a*	285·7		170·1	
Chlorophyll *b*	96·4		79·7	
Cell culture concentration (× 10^{-6}/ml)	7·9		6·1	
Volume/average cell (× 10^{-11} ml)	7·70		5·50	

TABLE 2. THE ABSORPTION MAXIMA OF CAROTENOIDS OF *Pedinomonas minor* AND *Pedinomonas tuberculata* IN VARIOUS SOLVENTS

Carotenoid	Petroleum spirit (40–60°)	Ethanol	Ethanol after HCl*	Chloroform	Carbon disulphide
α-Carotene	(418), 443, 473			(427), 456, 485	(448), 475, 506
β-Carotene	(423), 448, 476			(435), 463, 489	(450), 482, 509
Unidentified 1	(426), 452, 480				
Unidentified 2	(433), 454, 483			(441), 469, 498	
γ-Carotene	434, 457, 489			(445), 473, 504	(466), 494, 526
Unidentified 3	437, 463, 494			451, 478, 511	
Lycopene	442, 468, 500			456, 483, 517	478, 506, 544
Lutein	418, 443, 472	422, 445, 474	Nil	(428), 456, 486	(443), 472, 503
Zeaxanthin		(424), 450, 478	Nil	(430), 461, 490	
Lutein 5,6-epoxide	(419), 442, 470	(419), 444, 472	(398), 423, 448		
Violaxanthin	415, 438, 468	417, 441, 469	380, 401, 426	425, 450, 480	(440), 468, 500
Luteoxanthin		(377), 398, 421, 448	(360), 380, 401, 426	406, 429, 457	
Neoxanthin	412, 435, 464	(387), 413, 437, 466	(377), 398, 422, 449	420, 445, 475	
Neochrome		(377), 399, 421, 448	Nil	406, 429, 457	

Brackets indicate an approximate point of inflexion. Wavelengths in nm.

* Nil indicates little change of maxima after HCl treatment (final HCl concentration used 0·005 N).

The identities of lutein and lutein 5,6-epoxide were confirmed by the marked drop in relative polarity[6] after treatment with acid chloroform.[7]

The carotenes, α-, β- and γ-carotene and lycopene (all epiphasic to 95% aqueous methanol in petroleum spirit solution), were identified by comparison of their absorption maxima with those of authentic specimens, and by co-chromatography with authentic specimens on columns of magnesium oxide: Celite Hyflo-super-cel 1:1 w/w and also thin-layer silica-gel G plates, developing with petroleum spirit (b.p. 40–60°) containing acetone in both cases.

Table 3 shows the number of molecules of chlorophyll and carotenoid per average cell (which is the per plastid value, as these flagellates normally contain only one plastid per cell), the approximate unit membrane area per plastid and the number of molecules of chlorophyll and carotenoid per unit area of membrane. No account has been taken of the carotenoid content of the eyespot or possible extraplastidic pigment. The chlorophyll and carotenoid composition as percentages of cell dry weight (assuming dry weight to be 12 per cent of wet weight, and density of the cells to be one) is also shown in Table 3.

TABLE 3. THE CHLOROPHYLL AND CAROTENOID COMPOSITION OF THE PLASTIDS OF *Pedinomonas minor* AND *Pedinomonas tuberculata*

	P. minor	*P. tuberculata*
Approximate average cell dry weight (pg)*	9·24	6·6
Total chlorophyll (% of cell dry weight)	4·14	3·78
Total carotenoids (% of cell dry weight)	0·90	0·70
Chlorophyll per average plastid (molecules $\times 10^{-8}$)†	2·56	1·67
Carotenoids per average plastid (molecules $\times 10^{-7}$)†	9·29	5·20
Molar ratio chlorophylls:carotenoids	2·76	3·21
Approximate unit membrane area per plastid (μ^2)	531	396
Approximate molecular chlorophyll concentration (molecules $\times 10^{-5}$ per μ^2)	4·8	4·2
Approximate molecular carotenoid concentration (molecules $\times 10^{-5}$ per μ^2)	1·8	1·3

* Assuming dry weight to be 12 per cent of wet weight and density = 1.

† Assuming the mean molecular weights of chlorophylls and carotenoids to be 900 and 537 respectively.

Repetition using a different culture of *P. minor* gave three crude hydrocarbon fractions containing α-, β- and γ-carotene constituting approximately 5, 48 and 47 per cent respectively of the whole. The total carotene fraction appeared to be of similar magnitude to the xanthophyll fraction (although no separation was carried out to confirm this). Thus these results confirm those obtained in the initial investigation.

The absorption maxima values given for γ-carotene in Table 2 are for older samples, which also showed a *cis* peak at 348 nm in petroleum spirit solution. Freshly isolated samples however, were sometimes without the latter and showed absorption maxima shifted 1–2 nm to higher wavelengths.

DISCUSSION

Qualitatively the carotenoids of *P. minor* and *P. tuberculata* were not markedly different from those of the other Chlorophyceae described[8] except for the presence of lutein 5,6-epoxide

[6] N. I. KRINSKY, *Anal. Biochem.* **6**, 293 (1963).

[7] P. KARRER and E. LEUMANN, *Helv. Chim. Acta* **34**, 445 (1951).

[8] T. W. GOODWIN, In *Chemistry and Biochemistry of Plant Pigments* (Edited by T. W. GOODWIN), pp. 127–142. Academic Press, New York (1965).

in the former and lycopene in the latter. Lycopene has been isolated from antheridia of the Charophyceae.[9]

Quantitatively the results are noteworthy for the high concentration of γ-carotene in both flagellates, and particularly for the high total carotene concentration in *P. minor*, which was principally due to increased β- and γ-carotene concentrations. The latter flagellate also showed a low violaxanthin concentration compared to other green flagellates. The presence of small amounts of luteoxanthin and neochrome can probably be attributed either to breakdown of the 5,6-epoxides during processing, or to a small proportion of senescent cells in the original culture used.

Strain[10] has reported that many of the green algae which he investigated contained traces of γ-carotene. High concentrations of γ-carotene have been reported in the gametes of *Ulva*[10, 11] and in antheridia of Chara.[9] Krinsky and Levine[12] reported that *Chlamydomonas reinhardi* contained a *cis* isomer of γ-carotene to the extent of 2–4 per cent of total carotenoids. It is to be noted that only gametes have been found to have high concentrations of γ-carotene.

The flagellates are rich in chlorophylls and carotenoids, the percentage dry weight values being much higher than those reported in Goodwin.[13] The chlorophyll values resemble those reported in Rabinowitch[14] for *Chlorella* and the carotenoid values resemble those of Krinsky and Levine[12] for *Chlamydomonas*.

The chlorophyll *a*:*b* ratios differed somewhat in the two flagellates, being 3·0:1 and 2·1:1 in *P. minor* and *P. tuberculata* respectively. The molar ratios of chlorophyll to carotenoid were 2·8 and 3·2 respectively, and carotenes constituted 52 and 26 per cent of the total carotenoid in *P. minor* and *P. tuberculata* respectively.

The approximate molecular chlorophyll concentrations per unit area of plastid unit membrane are shown in Table 3 and are of the same order as the values found by Thomas *et al.*[15] for a variety of photosynthetic organisms (including green algae). By calculation from these results values of 208 Å^2 and 555 Å^2 for the areas available for a single chlorophyll or carotenoid molecule in a monolayer of a *P. minor* plastid were obtained. The corresponding values for *P. tuberculata* were 238 Å^2 and 769 Å^2. These chlorophyll results resemble those of Wolken and Schwertz[16] for two flagellates. Since the porphyrin part of the chlorophyll molecule is known to have an area of 225–242 Å^2 from X-ray studies, the results indicate that if the chlorophyll molecules were packed in a monolayer with the porphyrin rings in the same plane as the monolayer they would completely cover the entire unit membrane area. However, a consideration of the space available (assuming a sphere of maximum area calculated from the available cross-sectional area) and the electron-microscopically observed thickness of a unit membrane (approximately 70 Å in the case of *Pedinomonas*) indicates that sufficient membrane thickness is available for the chlorophyll molecules to lie at right angles or in some other orientation in relation to the plane of the membrane and still satisfy the size requirements of membrane. The value of less than 225 Å^2 obtained for the area available for

[9] P. KARRER, W. FATZER, M. FAVARGER and E. JUCKER, *Helv. Chim. Acta* **26**, 2121 (1943).
[10] H. H. STRAIN, In *Manual of Phycology* (Edited by G. M. SMITH), pp. 243–262. Chronica Botanica, Waltham, Mass. (1951).
[11] F. T. HAXO and K. A. CLENDENNING, *Biol. Bull.* **105**, 103 (1953).
[12] N. I. KRINSKY and R. P. LEVINE, *Plant Physiol.* **39**, 680 (1964).
[13] T. W. GOODWIN, In *Biochemistry and Physiology of Protozoa* (Edited by S. H. HUTNER), pp. 319–340. Academic Press, New York (1964).
[14] E. I. RABINOWITCH, In *Photosynthesis and Related Processes*, Vol. 1, pp. 409–413. Interscience, New York (1945).
[15] J. B. THOMAS, K. MINNAERT and P. F. ELBERS, *Acta Botan. Neerl.* **5**, 315 (1956).
[16] J. J. WOLKEN and F. A. SCHWERTZ, *J. gen. Physiol.* **37**, 111 (1953).

a single chlorophyll molecule to occupy may indicate (if the accuracy of membrane area estimation is sufficient, which seems doubtful) that the orientation of the chlorophyll molecules is not planar with respect to the membrane surfaces. The question of the orientation of chlorophyll molecules in plastids has been discussed by Thomas,[17] Wolken[18] and Clayton.[19] Recent work seems to indicate that the chlorophyll molecules are arranged in a non-planar way.

Taxonomically, the results indicate that these two *Pedinomonas* species are distinct from the other green flagellates so far described in their high γ-carotene content, and in the presence of lycopene and lutein 5,6-epoxide in *P. tuberculata* and *P. minor* respectively. They resemble other green flagellates in their possession of β-carotene, lutein, violaxanthin and neoxanthin. The carotenoid composition of the two organisms is not either qualitatively or quantitatively identical. The cultures were harvested at approximately the same phase of growth.

The results make a clear distinction between *Pedinomonas* and *Micromonas*.[20]

EXPERIMENTAL

Cultures

Pedinomonas tuberculata Vischer was a bacteria-containing unialgal culture which has been originally obtained from the Culture Collection of Algae and Protozoa, Cambridge (Strain No. 1965/2).

Pedinomonas minor (Strain 17B) was axenic and purified from a bacteria-containing unialgal culture provided by Dr. Hans Ettl.

Both cultures were grown under the conditions described in Ricketts[21] and harvested towards the end of the logarithmic phase of growth after about 3½ weeks' incubation at 14°. Cell numbers and packed cell volumes were determined as described in Ricketts.[5]

Pedinomonas minor would grow on the liquid medium solidified by the addition of 1% agar, whereas *P. tuberculata* would not. This indicates different growth requirements.

Pigments

The pigments were extracted into 90% aqueous acetone in the dark, extracted, saponified by treatment with 6% KOH in ethanol at 40° for 5 min in the dark under nitrogen,[12] and then partitioned as described in Ricketts[20] between petroleum spirit and aqueous methanol.

Chlorophyll assays were performed on aliquots of the original cultures using the method of Parsons and Strickland.[22]

Chromatography

This was in general carried out as described in Ricketts.[20] The carotene fraction was separated and purified by a combination of chromatography on alumina (British Drug Houses Ltd., for chromatography) and on magnesium oxide:Celite Hyflo-super-cel 1:1 (w/w); eluting with increasing concentrations of acetone in petroleum spirit (b.p. 40–60°) in both cases.

[17] J. B. THOMAS, In *Handbuch der pflanzenphysiologie* (Edited by W. RUHLAND), Vol. 5, part 1, pp. 548–552. Springer-Verlag, Berlin (1960).
[18] J. J. WOLKEN, In *Comparative Biochemistry of Photoreactive Systems* (Edited by M. B. ALLEN), pp. 145–168. Academic Press, New York (1960).
[19] P. K. CLAYTON, *Molecular Physics in Photosynthesis*, pp. 149–156. Blaisdell, New York (1965).
[20] T. R. RICKETTS, *Phytochem.* **5**, 571 (1966).
[21] T. R. RICKETTS, *Phytochem.* **4**, 725 (1965).
[22] T. R. PARSONS and J. D. H. STRICKLAND, *J. Marine Res.* **21**, 155 (1963).

The xanthophyll fractions were separated and purified by a combination of chromatography on icing sugar and on magnesium oxide: Celite Hyflo-super-cel 1:1 (w/w), eluting with increasing concentrations of acetone or ethanol in petroleum spirit (b.p. 40–60°).

Identification

Carotenes were identified by their positions on chromatographic columns, by their absorption spectra in at least two solvents, and by co-chromatography with authentic samples. Authentic samples of α- and β-carotene were purified from Sigma Chemical Co. β-carotene, Type III, from carrots. Authentic samples of γ-carotene and lycopene from *Blakeslea trispora* were kindly provided by Professor T. W. Goodwin and Dr. D. M. Thomas, of the University College of Wales, Aberystwyth.

Xanthophylls were also identified by their relative positions on column chromatography and absorption spectra in two solvents. In addition, 5,6-epoxides were detected by treating ethanolic solutions of the pigments with dilute aqueous hydrochloric acid to give a final concentration of 0·005 N. Lutein and lutein 5,6-epoxide were also identified by their marked drop in relative polarity after treatment with acid chloroform.[6, 7]

All absorption spectra were determined using an Optica Double-beam Grating Recording Spectrophotometer, CF4R. For the quantitative determination of the approximate pigment concentration of the unidentified pigments $E^{1\%}_{1cm}=2500$ was assumed. The extinction coefficients used for the carotenes were those given in Davies[23] and for the xanthophylls those in Krinsky and Levine.[12]

Relative polarities were determined by the method of Krinsky.[6]

The approximate unit membrane area per plastid was determined from electronmicrographs of ultra thin sections of the two flagellates embedded in Epon (kindly provided by Professor I. Manton, F.R.S.). In each case a doublet (terminology of Manton[24]) was counted as being equivalent to two unit membranes and the average number of unit membranes per plastid determined. Because of the shape of the plastids a number of assumptions had to be made. Firstly, that the average plastid size was 5·5 μ in length and 4·2 μ in maximum breadth in the case of *P. minor*, and 5·1 μ in length and 3·9 μ in maximum breadth in the case of *P. tuberculata*. (These values were obtained from average cell sizes, determined by photomicrography.) Secondly, that the area of a single membrane was 60 per cent of the area of an oblong with the sides the length and breadth of the plastid (calculated from the average shape of a plastid) and that all the membranes were of the same size. Knowing these values it was possible to calculate a value for the total unit membrane area of a plastid, and from this the number of pigment molecules per μ^2 unit membrane area. Obviously the approximations used in arriving at a value for the total plastid unit membrane area will probably lead to considerable errors in this value. Nevertheless the results are probably of the right order. Errors due to the assumption of unit size for each membrane will be balanced out to some extent by the curved nature of the membranes.

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[23] B. H. DAVIES, In *Chemistry and Biochemistry of Plant Pigments* (Edited by T. W. GOODWIN), pp. 489–532. Academic Press, New York (1965).
[24] I. MANTON, *J. Exp. Bot.* 13, 325 (1962).