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THE PIGMENT COMPOSITION OF SOME FLAGELLATES POSSESSING SCALY FLAGELLA

T. R. RICKETTS

Department of Botany, The University, Leeds, U.K.

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Abstract—The average cell pigment content of the green flagellates, *Platymonas chuii*, *Prasinocladus sp.* and *Micromonas squamata* has been investigated. *Platymonas chuii* contained α-carotene, β-carotene, γ-carotene, unidentified carotene, lycopene, lutein, zeaxanthin, lutein 5,6-epoxide, violaxanthin, ?trollein, neoxanthin, chlorophyll *a* and chlorophyll *b* in the following amounts per average cell: 3·7, 67·0, 19·7, 2·5, 5·2, 180·3, 76·3, 22·4, 68·0, 74·7, 49·6, 1403·6, and 618·6 pg (× 1000). *Prasinocladus sp.* contained the same carotenoids (but no unidentified carotene) in the following amounts: 9·8, 60·4, 13·4, 9·3, 104·3, 10·7, 21·5, 79·1, 22·3, 62·5, 1188·4 and 782·4 pg (× 1000) per average cell. The values are given in the same order as described above for *Platymonas*. *Micromonas squamata* contained α-carotene, β-carotene, two unidentified carotenes, monohydroxy-5,6-epoxy-α-carotene, micronone, two unidentified xanthophylls, violaxanthin, four unidentified xanthophylls, neoxanthin, chlorophyll *a* and chlorophyll *b* in the following amounts per average cell: 0·25, 2·26, 0·06, 0·05, 1·21, 6·43, 1·39, 0·70, 1·75, 0·38, 1·43, 3·00, 0·42, 4·40, 70·2 and 37·7 pg (× 1000). Older cultures contained a more polar ketonic xanthophyll in addition which replaced micronone as the principal xanthophyll. The possible identities of the unnamed carotenoids are discussed. It is to be noted that these organisms possess a single plastid. The per average cell values are therefore identical with the per average plastid values. The taxonomic significance of the findings is discussed. Those flagellates already investigated which bear scaly flagella may be divided into two distinct groups on a pigment composition basis.

INTRODUCTION

THE phytoflagellates *Micromonas squamata*,[1] *Nephroselmis gilva*,[2] *Pyramimonas* and *Halosphaera spp.*,[3] *Prasinocladus marinus*,[4] *Heteromastix spp.*,[5] *Platymonas spp.*[6] and *Mesostigma viride*[7] have all been shown to possess scale-bearing flagella. They have been assigned to the Prasinophyceae[6] (*sensu* Christensen[8]) as a new class to accommodate non-Volvocalean monads containing chlorophyll *b*. Several of these organisms (*Micromonas*, *Heteromastix* and *Pyramimonas*) have been shown to contain an unusual pigment resembling protochlorophyll, magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester.[9] This pigment could not be demonstrated in one species (out of the three examined) of *Heteromastix*. None was found in a number of other green flagellates. The other flagellates possessing scaly flagella have not yet been examined.

Preliminary results also indicated that all the organisms containing the protochlorophyll-like pigment had unusual carotenoid compositions, containing one or more orange-red

[1] I. MANTON and M. PARKE, *J. Marine Biol. Ass. U.K.* **39**, 275 (1960).
[2] M. PARKE and D. G. RAYNS, *J. Marine Biol. Ass. U.K.* **44**, 209 (1964).
[3] I. MANTON, K. OATES and M. PARKE, *J. Marine Biol. Ass. U.K.* **43**, 225 (1963).
[4] M. PARKE and I. MANTON, *J. Marine Biol. Ass. U.K.* **45**, 525 (1965).
[5] I. MANTON, D. G. RAYNS, H. ETTL and M. PARKE, *J. Marine Biol. Ass. U.K.* **45**, 241 (1965).
[6] I. MANTON and M. PARKE, *J. Marine Biol. Ass. U.K.* **45**, 743 (1965).
[7] I. MANTON and H. ETTL, *J. Linn. Soc. (Bot.)* **59**, 175 (1965).
[8] T. CHRISTENSEN. In *Botanik* (Edited by T. W. BOCHER, M. LANGE and T. SØRENSEN), Bd. 2 (Systematisk Botanik), Nr. 2, p. 128. Munksgaard, Copenhagen (1962).
[9] T. R. RICKETTS, *Phytochem.* **5**, 223 (1966).

pigments as the major xanthophylls.[9] A more detailed investigation of the type species of Micromonas, *Micromonas pusilla* (Butcher) (an organism which does not itself bear scales upon its flagellum[10]), showed that this flagellate had an hitherto undescribed type of carotenoid composition.[11] The main xanthophyll was an undescribed keto-xanthophyll, of probable structure monohydroxy, monoketo-6′ (and/or 5′)-hydro-5,8-epoxy-β-carotene, for which the name micronone was proposed. This constituted about 45 per cent of total carotenoids.

Preliminary results[9] on other related genera, notably *M. squamata*, some *Heteromastix spp.* and *Pyramimonas amylifera* indicated that all these had carotenoid compositions similar to, if not identical with, that of *M. pusilla*. The present work was undertaken to extend the pigment analyses to more flagellar-scale-bearing organisms in order to try to elucidate their phyletic relationships, both within the group and outside it.

RESULTS

The pigment composition of *Platymonas chuii* is shown in Table 1. No protochlorophyll-like pigment could be demonstrated in this organism, using either the method of Parsons[9, 12] or that of Granick.[13] Xanthophyll F3 gave absorption spectra and maxima (ethanol, (421), 444, 471 nm; chloroform, (428), 454, 482 nm; carbon disulphide, (445), 472, 501·5 nm) which resembled those of lutein and its derivatives. Addition of aqueous hydrochloric acid

TABLE 1. THE PIGMENT COMPOSITION OF *Platymonas chuii*. THE CAROTENOIDS ARE IN ORDER OF INCREASING ADSORPTION

Fraction	Identification	% of total carotenoids	pg/average cell (×1000)
Plα	α-carotene	0·7	3·7
Plβ	β-carotene	11·7	67·0
P2	γ-carotene	3·5	19·7
P2A	unidentified	0·4	2·5
P3	lycopene	0·9	5·2
Fl	lutein	31·7	180·3
F1Z	zeaxanthin	13·4	76·3
F1A	lutein 5,6-epoxide	3·9	22·4
F2	violaxanthin	11·9	68·0
F3	?trollein	13·1	74·7
F4	neoxanthin	8·7	49·6
	Total carotenoids	99·9	569·4
	chlorophyll *a*		1403·6
	chlorophyll *b*		618·6
	Total chlorophylls		2022·2
Average values per cell	Volume		$7{\cdot}92 \times 10^{-10}$ ml
	Approximate wet weight (calculated)		792 pg
	Approximate dry weight (calculated)		95·2 pg
	Chlorophyll as % dry weight		2·1
	Carotenoid as % dry weight		0·6
	Culture cell concentration ($\times 10^{-6}$/ml) when harvested		0·42

[10] I. MANTON, *J. Marine Biol. Ass. U.K.* **38**, 319 (1959).
[11] T. R. RICKETTS, *Phytochem.* **5**, 571 (1966).
[12] T. R. PARSONS, *J. Marine Res.* **21**, 164 (1963).
[13] S. GRANICK, *J. Biol. Chem.* **183**, 713 (1950).

produced no shift in absorption maxima, indicating the absence of 5,6-epoxide groupings. The M_{50} value was 57·3 per cent, indicating a relative polarity of 2·82.

Carotene P2A showed absorption maxima at 436, 461 and 493 nm in light petroleum (b.p. 40–60°).

The pigment composition of *Prasinocladus sp.* is shown in Table 2. This much resembles the results obtained for *Platymonas chuii.* No protochlorophyll-like pigment could be demonstrated in the flagellate using the method of estimation of Parsons[9, 12]. Fraction F4 of *Prasinocladus* was identical with Fraction F3 of *Platymonas chuii.*

TABLE 2. THE PIGMENT COMPOSITION OF *Prasinocladus sp.* (PLYMOUTH NO. 371). THE CAROTENOIDS ARE IN ORDER OF INCREASING ADSORPTION

Fraction	Identification	% of total carotenoids	pg/average cell (×1000)
P1A	α-carotene	2·5	9·8
P1B	β-carotene	15·3	60·4
P2	γ-carotene	3·4	13·4
P3	lycopene	2·4	9·3
F1L	lutein	26·5	104·3
F1Z	zeaxanthin	2·7	10·7
F2	lutein 5,6-epoxide	5·5	21·5
F3	violaxanthin	20·1	79·1
F4	?trollein	5·7	22·3
F5	neoxanthin	15·9	62·5
	Total carotenoids	100·0	393·3
	chlorophyll *a*		1188·4
	chlorophyll *b*		782·4
	Total chlorophylls		1970·8
Average values per cell	Volume		$7{\cdot}46\times10^{-10}$ ml
	Approximate wet weight (calculated)		746 pg
	Approximate dry weight (calculated)		89·5 pg
	Chlorophyll as % dry weight		2·2
	Carotenoid as % dry weight		0·44
	Culture cell concentration ($\times10^{-6}$/ml) when harvested		0·19

The pigment composition of *Micromonas squamata* is shown in Table 3. The protochlorophyll-like pigment content of this culture was not estimated, but would be about 5 per cent of the total chlorophylls in magnitude, extrapolating from previous results.[9] The carotenoid composition is basically very similar to that of *M. pusilla.*[11] Fractions F2, F3 and F7 proved to be identical with micronone, unidentified xanthophylls F2B1 and F6 respectively of *M. pusilla.*[11] Absorption maxima and relative polarities of the unidentified carotenoids are shown in Table 4.

Xanthophyll F4 was a 5,6-epoxide which resembled lutein 5,6-epoxide in its absorption maxima and relative polarity.

Fraction F6A was another 5,6-monoepoxide. The absorption spectra of this xanthophyll resembled that of 5,6-epoxides of α-carotene. The relative polarity (2·38) indicated that it was not lutein 5,6-epoxide (r.p. = 2·13) or trollixanthin (r.p. = 3·13?).

Fraction F6B appears to be the 5,8-epoxide of xanthophyll F6A. It showed an almost identical relative polarity, the same R_f using thin-layer chromatography on silica-gel G, and almost identical absorption spectra when compared with the 5,8-epoxide of Fraction F6A.

TABLE 3. THE PIGMENT COMPOSITION OF *Micromonas squamata*. CAROTENOIDS GIVEN IN ORDER OF INCREASING ADSORPTION

Fraction	Identification	% of total carotenoids	pg/average cell (× 1000)
P1α	α-carotene	1·1	0·25
P1β	β-carotene	9·5	2·26
P1A	unidentified carotene	0·3	0·06
P2	unidentified carotene	0·2	0·05
F1	monohydroxy-5,6-epoxy-α-carotene	5·1	1·21
F2	micronone	27·1	6·43
F3	unidentified	5·9	1·39
F4	unidentified	2·9	0·70
F5	violaxanthin	7·4	1·75
F6A	unidentified	1·6	0·38
F6B	unidentified	6·0	1·43
F6C	unidentified	12·6	3·00
F7	unidentified	1·8	0·42
F8	neoxanthin	18·5	4·40
	Total carotenoids		23·73
	chlorophyll *a*		70·2
	chlorophyll *b*		37·7
	Total chlorophylls		107·9
Average values per cell	Volume		$3{\cdot}3 \times 10^{-11}$ ml
	Approximate wet weight (calculated)		33·0 pg
	Approximate dry weight (calculated)		3·96 pg
	Chlorophyll as % dry weight		2·7
	Carotenoid as % dry weight		0·6
	Culture cell concentration ($\times 10^{-6}$/ml) when harvested		3·0

TABLE 4. THE ABSORPTION MAXIMA OF THE UNIDENTIFIED CAROTENOIDS OF *Micromonas squamata* IN VARIOUS SOLVENTS

Fraction	Light petroleum (40–60°)	Chloroform	Ethanol*	M_{50}	Relative polarity
P1A	(418), 442, 471	(426), 452, 481			
P2	(453), 477, 514	485			
F3	(398), 424, 449	410, 436, 461	(404), 427, 452	81·4	1·91
F4		432, 456, 483	(422), 447, 471	76·0	2·11
F4 after HCl*			403, 426, 452		
F6A		453, 477	442, 467	68·8	2·38
F6A after HCl*			(403), 426, 452		
F6B		433, 458	(403), 427, 450	70·0	2·34
F6C	(398), 423, 448	432, 458	422, 448	68·6	2·38
F7	(400), 422, 448	(408), 433, 459	(404), 424, 450	55·0	2·79

* All these xanthophyll solutions were treated with N HCl to a final concentration of 0·005 N. In all cases except F4 and F6A the maxima were virtually unchanged after this treatment.

Brackets indicate the approximate position of a point of inflexion.

Fraction F6C resembled Fraction F6B in relative polarity and absorption maxima but showed a greater R_f on silica-gel G thin-layer chromatography. It may be closely related in structure or could be a zeacarotene derivative.

Fraction F7 displayed absorption spectra similar to those of Fraction F6 of *M. pusilla*.[11] The maxima were at slightly lower wavelengths. (This is probably a function of the difference in spectrophotometers used in the estimations. In general the Optica CF4R spectrophotometer gives maxima at 1–2 nm lower in wavelength compared with the machine previously used.[11]) The two xanthophylls had the same R_f on silica-gel G thin-layer chromatography. Fraction F7 showed the same results on treatment with acid chloroform[14] as Fraction F6.[11] The relative polarity (2·79) was the same within the experimental error of the method.[15] It was therefore concluded that the two were identical.

Older cultures of *M. squamata* displayed a considerable change in xanthophyll composition. The main xanthophyll, micronone, was reduced in percentage to about a quarter of its previous value. In its place there appeared a new, more polar, ketonic xanthophyll, which constituted about 16 per cent of total carotenoids. Details of micronone and the more polar unidentified ketonic xanthophyll are given in Table 5. The single absorption maximum of the latter in ethanol compared with the triple absorption maxima in light petroleum indicates that this xanthophyll molecule contains a conjugated carbonyl grouping. The increase in relative polarity of 0·18 on borohydride reduction[15] indicates the reduction of a single ketonic grouping (r.p. = 0·72) to an allylic hydroxyl grouping (r.p. = 0·89).

TABLE 5. THE ABSORPTION MAXIMA OF THE KETO-XANTHOPHYLLS OF *Micromonas squamata* IN VARIOUS SOLVENTS, AND AFTER BOROHYDRIDE REDUCTION

Fraction	Light petroleum (40–60°)	Ethanol	Chloroform	M_{50}	Relative polarity	% of total carotenoids
F2 (micronone)	(418), 440, 468	445	454	78·2	2·03*	7·1
F2 reduced		(375), 396, 419, 446				
F6X	(429), 452, 480	452	465	66·2	2·48	16·4
F6X reduced		(375), 399, 421, 447		60·0	2·66	

* Values for *Micromonas pusilla* micronone.[11]
Brackets indicate the approximate position of a point of inflexion.

DISCUSSION

The Identity of the Carotenoids

Platymonas chuii. The xanthophyll F3 had spectral and adsorptive properties resembling those described for trollein in orange juice.[16,17] Curl and Bailey[16] suggested that the xanthophyll possessed at least three hydroxyl groupings. Strain[18] described the spectral and adsorptive properties of an unnamed xanthophyll present as a minor pigment in many green algae. This was probably trollein. A pigment identified as trollein has been described in *Euglena*[19] and *Chlamydomonas reinhardi*[20] as a minor xanthophyll constituent. Krinsky[15] found that

[14] F. J. PETRACEK and L. ZECHMEISTER, *J. Am. Chem. Soc.* **78**, 1427 (1956).
[15] N. I. KRINSKY, *Anal. Biochem.* **6**, 293 (1963).
[16] A. L. CURL and G. F. BAILEY, *J. Agr. Food Chem.* **2**, 685 (1954).
[17] A. L. CURL and G. F. BAILEY, *Food Res.* **20**, 371 (1955).
[18] H. H. STRAIN, *Chloroplast Pigments and Chromatographic Analysis*, pp. 39–40. Pennsylvania University, Penn. (1958).
[19] N. I. KRINSKY, A. GORDON and A. I. STERN, *Plant Physiol.* **39**, 441 (1964).
[20] N. I. KRINSKY and R. P. LEVINE, *Plant Physiol.* **39**, 680 (1964).

this xanthophyll had a relative polarity of 2·98 (M_{50} 53·3%) which did not alter after treatment with acidic methanol. He concluded that the pigment was a trihydroxy-xanthophyll possessing no allylic hydroxyl groupings. Relative polarity determinations on the pigment described as ?trollein in the present work gave a value of 2·82. This value is just about the same as that obtained by Krinsky[15] (2·98) taking into account the experimental error of the method, estimated to be ±5 per cent by this author.[15]

Unfortunately insufficient material is available at the present time to permit structural investigations.

Prasinocladus sp. The analytical results for this flagellate are qualitatively very similar to those obtained with *Platymonas chuii.* Quantitatively there are differences, but in the main, the proportions of the carotenoids are relatively similar. The relative proportions of the pigments may well depend upon the time of harvesting in relation to the phase of growth and nutritive conditions.

Micromonas squamata. Fraction F2 (micronone) and unidentified Fractions F3 and F7 proved to be identical with *M. pusilla* xanthophylls. Their possible structures have already been discussed.[11]

Fractions F4, F6A, F6B and F6C have been discussed to some extent in the Results Section. They have not been identified. Fraction F4 was a 5,6-epoxide, similar to lutein 5,6-epoxide. Fraction F6A also appeared to be a 5,6-monoepoxide of α-carotene, with a somewhat greater relative polarity than Fraction F4. Fraction F6B was the 5,8-epoxide of Fraction F6A. Fraction F6C may be related in structure to Fraction F6B or may possibly be a zeacarotene derivative. The ketonic xanthophyll Fraction F6X, which is found in older cultures of *M. squamata* (Table 5), does not appear to be identical with any described xanthophyll. The shape of the absorption spectra and the position of maximum absorption of the reduced pigment compared with that of microxanthin,[11] together with the fact that there is a concurrent disappearance of micronone and an appearance of Fraction F6X in older cultures suggest that micronone and the latter have related structures.

GENERAL DISCUSSION

Platymonas chuii and *Prasinocladus sp.* display a similar and relatively normal chlorophycean pigment composition. The results obtained are supporting evidence for their close relationship to one another. They show traces of γ-carotene and lycopene. The presence of these carotenes has been reported in much larger amounts in the green flagellate genus Pedinomonas.[21] The occurrence of γ-carotene in green algae is discussed in the same paper. The xanthophyll fractions are noteworthy only for the relatively high concentration of a pigment provisionally identified as trollein. As mentioned earlier trollein has been demonstrated in *Euglena, Chlamydomonas* and a number of other green algae.

Micromonas squamata displays a pigment composition which is basically very similar to that found in *M. pusilla,*[11] and supports their close relationship. The main differences between the two were the absence of the ketonic xanthophyll F5B and xanthophyll F2B2 of *M. pusilla* from *M. squamata,* and the presence of xanthophylls F4, F6A, F6B and F6C in the latter, which were not demonstrated in the former.

The results show clearly that flagellates possessing scaly flagella do not all have identical qualitative carotenoid compositions. Two distinct types of composition have so far been demonstrated. The first type, shown by *Platymonas chuii* and *Prasinocladus sp.*, very much

[21] T. R. RICKETTS, *Phytochem.* 6, 19 (1967).

resembles the normal chlorophycean pattern. The second type, shown by *M. squamata*, has only been described previously in the related *M. pusilla*[11] (an organism with a single naked flagellum). It is to be noted that the presence of these unusual xanthophylls has so far been found to parallel the presence of magnesium 2,4 divinylphaeoporphyrin a_5 monomethyl ester[9] in green flagellates. It thus appears probable that a more detailed investigation of *Heteromastix* and *Pyramimonas* (both displaying the protochlorophyll-like pigment) will confirm the preliminary observations[9, 11] that these flagellates have ketonic xanthophylls as the main carotenoid.

It would appear that the results rule out a very close phyletic relationship between *Micromonas* and the other two genera, unless the possession of flagellar scales were a more primitive character than plastid pigment composition. In that case a recent modification in pigment character must have occurred. However the pigment compositions encountered are so dissimilar that this seems unlikely.

It is hoped to extend the pigment analyses to the remaining unexamined scaly flagellates in the near future.

EXPERIMENTAL

Cultures

Platymonas chuii Butcher (Cambridge Culture Collection No. 8/6, listed as *Tetraselmis chuii* Butcher) was available as a bacteria-containing unialgal culture. It was rendered bacteria-free by a plating technique using Erdschrieber medium made with 50% v/v aqueous sea-water and solidified by the addition of 1% w/v agar. Electron microscopic examination of ultra-thin sections indicated that this organism was a *Platymonas sp.* (Professor I. Manton, F.R.S., personal communication). The flagellate was grown axenically in 100 ml aliquots of Erdschrieber medium made using 50% v/v sea-water in place of 100% v/v sea-water (Dr. E. A. George, personal communication) for pigment assay.

Micromonas squamata (Plymouth Collection No. 290) was grown in bacteria-containing culture in 1·5 l. aliquots of Erdschrieber medium. (This flagellate was originally designated *Thalassomonas pusilla* Butcher, but more recent electron-microscopic examinations[2] have shown the organism to be indistinguishable from *M. squamata*.)

Prasinocladus sp. (Plymouth No. 371) was obtained from Dr. Mary Parke, of the Plymouth Laboratory. The axenic culture originated from Dr. L. Provasoli of the Haskins Laboratories, New York. It was grown in the same way as *Platymonas* in axenic culture. Its identity as a *Prasinocladus sp.* was confirmed electron-microscopically (Professor I. Manton, F.R.S., personal communication). This organism had a great tendency to become non-motile in culture and adhere to culture vessels. For this reason it was impossible to denote the phase of growth at harvest. The cultures harvested showed about 20–30 per cent of motile cells, which was about the level of motile cells present throughout growth.

All cultures were incubated at 14° with a regimen of 16 hr light (200 lm/ft^2) and 8 hr darkness per day. The cultures were harvested towards the end of the logarithmic phase unless specified otherwise. Cell numbers and packed cell volumes were determined as described in Ricketts[22] for *Pedinomonas*. With *Prasinocladus* a white layer, consisting principally of empty thecae, was found uppermost on centrifugation. It had about 40 per cent of the volume of the packed cells and was not included in the packed cell volume determination. The approximate cell dry weights were calculated from the packed cell volumes

[22] T. R. Ricketts, *Phytochem.* **5**, 67 (1966).

by assuming the dry weight to be 12 per cent of the wet weight. The latter was calculated by assuming a density of one for the packed cells.

Pigments

The pigments were extracted with 90% v/v aqueous acetone from the packed cell deposit and then saponified and partitioned as described in Ricketts.[21] In the cases of *Platymonas* and *Prasinocladus* the pigment was not easily extractable from the cells and grinding with sand proved to be necessary for complete extraction (ultrasonic treatment or alternate freezing and thawing were less successful). Chlorophyll assays were carried out as in Ricketts.[21]

Chromatography

This was carried out as described in Ricketts.[21] Additionally the purity of the carotenes and xanthophylls was checked by thin-layer chromatography on silica gel G, developing with light petroleum (b.p. 40–60°) containing 0·75% v/v or 30% v/v acetone respectively. These would not separate α- and β-carotene or lutein and zeaxanthin respectively.

Identification

The methods and extinction coefficients used were as described in Ricketts.[21] In addition, co-chromatography of certain of the pigments here described with the lutein, lutein 5,6-epoxide, violaxanthin and neoxanthin of *Pedinomonas*,[21] and with certain of the xanthophylls of *M. pusilla*[11] was carried out on thin-layer silica gel G, developing with light petroleum (b.p. 40–60°) containing 30% v/v acetone. An Optica Double-beam Grating Recording Spectrophotometer, CF4R, was used for the spectrophotometric determinations.

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