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FURTHER INVESTIGATIONS INTO THE PIGMENT COMPOSITION OF GREEN FLAGELLATES POSSESSING SCALY FLAGELLA

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Abstract—The average cell pigment content of the phytoflagellates *Nephroselmis gilva*, *Heteromastix longifilis*, *Pyramimonas amylifera* and *P. obovata* have been investigated. *N. gilva* contained α-carotene, β-carotene, monohydroxy, mono-5,6-epoxy-α-carotene, micronone, lutein, unidentified xanthophyll B, lutein-5,6-epoxide, violaxanthin, unidentified xanthophyll K and neoxanthin in the following amounts per average cell: 0·5, 10·9, 3·6, 5·2, 0·8, 7·1, 1·9, 4·6, 22·3 and 15·5 pg (× 1000) respectively in saponified extracts. The chlorophyll *a*, *b* and magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester contents per average cell were 133·9, 176·6 and 7·5 pg (× 1000) respectively. *H. longifilis* contained α-carotene, β-carotene, γ-carotene, lycopene, unidentified xanthophyll A, zeaxanthin, lutein-5,6-epoxide, violaxanthin, siphonein, neoxanthin, unidentified xanthophyll K2, chlorophyll *a*, chlorophyll *b* and magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester in the following amounts per average cell: 2·3, 4·8, 2·1, 1·0, 0·3, 4·0, 0·1, 1·2, 6·5, 4·4, 3·4, 65·7, 48·6 and 1·1 pg (×1000) respectively. *P. amylifera* contained α-carotene, β-carotene, γ-carotene, zeaxanthin, lutein-5,6-epoxide, violaxanthin, siphonein, neoxanthin, unidentified xanthophyll K2, chlorophyll *a*, chlorophyll *b* and magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester in the following amounts per average cell: 49, 551, 8, 673, 34, 130, 404, 836, 228, 11772, 6937, and 403 pg (×1000) respectively. *P. obovata* contained α-carotene, β-carotene, γ-carotene, unidentified carotene, lutein, zeaxanthin, violaxanthin, ? trollein, neoxanthin, chlorophyll *a* and chlorophyll *b* in the following amounts per average cell: 2, 49, 6, 1, 28, 30, 18, 18, 19, 324 and 335 pg (×1000) respectively. The taxonomic significance of these findings is discussed. The finding of siphonein and a more polar, but closely related, xanthophyll K2 in *H. longifilis* and *P. amylifera* indicates affinities with the siphonalean green algae. The flagellates possessing scaly flagella may be divided into three groups on a pigment basis composition. This provides another means of further subdividing the Prasinophyceae.

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 (scaly) flagella. They have been assigned to the Prasinophyceae[6] (sensu Christensen,[8] as a new class to accommodate non-Volvocalean monads containing both chlorophylls *a* and *b*).

Several of the flagellates (*Micromonas*, *Heteromastix* and *Pyramimonas*) have been shown to contain an unusual pigment, magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester.[9] This pigment could not be demonstrated in one species (out of the three

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[1] I. MANTON and M. PARKE, *J. Marine Biol. Assoc. U.K.* **39**, 275 (1960).
[2] M. PARKE and D. G. RAYNS, *J. Marine Biol. Assoc. U.K.* **44**, 209 (1964).
[3] I. MANTON, K. OATES and M. PARKE, *J. Marine Biol. Assoc. U.K.* **43**, 225 (1963).
[4] M. PARKE and I. MANTON, *J. Marine Biol. Assoc. U.K.* **45**, 525 (1965).
[5] I. MANTON, D. G. RAYNS, H. ETTL and M. PARKE, *J. Marine Biol. Assoc. U.K.* **45**, 241 (1965).
[6] I. MANTON and M. PARKE, *J. Marine Biol. Assoc. U.K.* **45**, 743 (1965).
[7] I. MANTON and H. ETTL, *J. Linn. Soc.* (*Botany*) **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 (1966).
[9] T. R. RICKETTS, *Phytochem.* **5**, 223 (1966).

examined) of *Heteromastix*. Later work[10] showed that the pigment could not be demonstrated in *Platymonas chuii* or in a *Prasinocladus* sp.

Preliminary results[9] had indicated that all the flagellates showing the protochlorophyll-like pigment (magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester) had unusual carotenoid compositions, containing one or more orange-red xanthophylls as the major xanthophyll components.

A more detailed investigation of *M. pusilla* (an organism showing the protochlorophyll-like pigment but possessing no flagellar scales[11]) showed that this flagellate had an hitherto undescribed type of carotenoid composition.[12] The main xanthophyll isolated was a new carbonyl-containing xanthophyll, of suggested structure monohydroxy, monoketo-6'-(and/or5')-hydro-5,8-epoxy-β-carotene, for which the name micronone was proposed. This constituted 45 per cent of the total carotenoids. An investigation of *M. squamata*[10] (an organism bearing flagellar scales) showed a xanthophyll composition essentially similar to that of *M. pusilla*. In older cultures of *M. squamata* the proportion of micronone was reduced and a more polar ketonic xanthophyll appeared. *Platymonas chuii* and a *Prasinocladus* sp. showed a quite dissimilar carotenoid pattern[10] which resembled the normal chlorophycean type. Thus two types of carotenoid composition have so far been described in the green scaly flagellates. The present work extends the range of pigment analyses to cover other representatives of the scaly flagellate family: *Heteromastix* spp., *Pyramimonas* spp. and *Nephroselmis* spp. The first two of these genera have been shown to be likely to have xanthophyll compositions resembling that of *Micromonas* by preliminary investigations.[9]

RESULTS

(*a*) *Pigment Composition*

The pigment composition of *Nephroselmis gilva* is shown in Table 1. It shows a great resemblance to that of the genus *Micromonas*.[10, 12]

Xanthophyll F1 was a 5,6-monoepoxide showing a r.p. (relative polarity; see Experimental Section) of 1·38. This indicated that the molecule probably contained an hydroxyl grouping in addition to the monoepoxide grouping. It showed the same R_f's on polyamide TLC (thin-layer chromatography) as xanthophyll F1 of *M. squamata*[10] (Table 6). Its spectral characteristics (chloroform; (427),451,479 nm: ethanol; (418),442,470 nm: ethanol; (400),423,448 nm after HCl treatment) and r.p. indicate that it is probably a monohydroxy, mono-5,6-epoxy derivative of α-carotene.

Micronone,[12] from *N. gilva*, showed the same R_f's on polyamide TLC (Table 5) as those obtained with the *Micromonas* pigment. Its r.p. of 2·07 increased to 2·38 on borohydride reduction (and after subsequent purification). This change would be consistent with reduction of a single ketone grouping to a non-allylic hydroxyl grouping (0·28 change) or with the reduction of two carbonyl groupings to two allylic hydroxyl groupings (0·34 change in r.p.). Almost complete reduction was obtained in 30 min. Absorption maxima and other properties are given in Table 5.

Details of the chromatographic properties of unidentified xanthophyll B are given in Table 6. These were identical with those of xanthophyll F2B1 and xanthophyll F3 of *M. pusilla*[12] and *M. squamata*[10] respectively. The spectral properties of xanthophyll B (chloroform; 435,461 nm: petroleum spirit (40–60°); (399),424,449 nm: ethanol; 426,451 nm) and

[10] T. R. RICKETTS, *Phytochem.* **6**, 669 (1967).
[11] I. MANTON, *J. Marine Biol. Assoc. U.K.* **38**, 319 (1959).
[12] T. R. RICKETTS, *Phytochem.* **5**, 571 (1966).

TABLE 1. THE PIGMENT COMPOSITION OF *Nephroselmis gilva**

Fraction	Identification	% of total carotenoids	pg/average cell (×1000)
P1A	α-carotene	0·7	0·53
P1B	β-carotene	15·1	10·94
F1	monohydroxy, mono-5,6-epoxy-α-carotene	4·9	3·57
F2	micronone	7·2	5·20
F3X	lutein	1·1	0·78
F3	unidentified xanthophyll B	9·9	7·15
F4	lutein-5,6-epoxide	2·6	1·85
F5	violaxanthin	6·4	4·60
F6	unidentified xanthophyll K	30·8	22·27
F7	neoxanthin	21·4	15·47
	Total carotenoids	100·0	72·36
	Chlorophyll *a*		133·9
	Chlorophyll *b*		176·6
	Magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester		7·5
	Total chlorophylls		318·0

* The culture harvested had a cell concentration of $0{\cdot}88 \times 10^6$ cells/ml. The average cell values were as follows: volume, $1{\cdot}53 \times 10^{-10}$ ml; approximate calculated wet weight, 153 pg; approximate calculated dry weight 18·4 pg. Chlorophylls and carotenoids constituted 1·7 and 0·4 per cent respectively of the cell dry weight. The carotenoid composition was investigated only with saponified pigment extracts.

TABLE 2. THE PIGMENT COMPOSITION OF *Pyramimonas obovata**

Fraction	Identification	% of total carotenoids	pg/average cell (×1000)
P1A	α-carotene	1·0	1·7
P1B	β-carotene	28·4	48·5
P2	γ-carotene	3·6	6·1
P3	unidentified carotene	0·8	1·3
F1L	lutein	16·3	27·8
F1Z	zeaxanthin	17·6	30·1
F2	violaxanthin	10·4	17·8
F3	? trollein	10·6	18·1
F4	neoxanthin	11·4	19·4
	Total carotenoids		170·8
	Chlorophyll *a*		324·1
	Chlorophyll *b*		335·0
	Total chlorophylls		659·1

* The culture harvested had a cell concentration of $0{\cdot}5 \times 10^6$ cells/ml. The average cell values were as follows: volume, $2{\cdot}2 \times 10^{-10}$ ml; Approximate calculated wet weight, 220 pg; approximate calculated dry weight, 26·4 pg. Chlorophylls and carotenoids constituted 2·5 and 0·6 per cent respectively of cell dry weight.

r.p. of 1·93 are also in good agreement with those of xanthophylls F2B1 and F3 (r.p.'s of 1·96 and 1·91 respectively; absorption maxima).[10, 12] The three were therefore considered to be identical.

Unidentified xanthophyll K was the major carotenoid constituent isolated. Details of its absorption maxima, chromatographic properties and r.p. together with those of its reduction product are shown in Table 5. These were identical with those of xanthophyll

TABLE 3. THE PIGMENT COMPOSITION OF *Pyramimonas amylifera**

Fraction	Identification	% of total carotenoids	pg/average cell (× 1000)
P1A	α-carotene	1·7	49·1
P1B	β-carotene	18·9	550·9
P2	γ-carotene	0·3	8·2
F1	zeaxanthin	23·1	672·9
F2	lutein 5,6-epoxide	1·2	33·7
F3	violaxanthin	4·4	129·7
F4	neoxanthin	13·9	404·0
F5	xanthophyll K1S (siphonaxanthin)†	28·7	835·9
F6	unidentified xanthophyll K2S†	7·8	227·7
	Total carotenoids	100·0	2912·1
	Chlorophyll *a*		11,772
	Chlorophyll *b*		6937
	Magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester		403
	Total chlorophylls		19,112

* The culture harvested had a cell concentration of $0{\cdot}05 \times 10^6$ cells/ml. The average cell values were as follows: volume, $2{\cdot}95 \times 10^{-9}$ ml; approximate calculated wet weight, 2950 pg; approximate calculated dry weight, 354 pg. Chlorophylls and carotenoids constituted 5·4 and 0·8 per cent respectively of cell dry weight.

† Isolated from saponified extracts. Present in unsaponified cells as siphonein (xanthophyll K1) and xanthophyll K2.

TABLE 4. THE PIGMENT COMPOSITION OF *Heteromastix longifilis**

Fraction	Identification	% of total carotenoids	pg/average cell (× 1000)
P1	α-carotene	7·6	2·27
P2	β-carotene	16·0	4·81
P3	γ-carotene	6·9	2·06
P4	lycopene	3·1	0·95
P5	unidentified xanthophyll A	1·0	0·31
F1	zeaxanthin	13·3	3·99
F2	lutein 5,6-epoxide	0·4	0·12
F3	violaxanthin	4·1	1·22
F4	neoxanthin	14·7	4·42
F5	xanthophyll K1S (siphonaxanthin)†	21·7	6·52
F6	unidentified xanthophyll K2S†	11·2	3·36
	Total carotenoids	100·0	30·03
	Chlorophyll *a*		65·67
	Chlorophyll *b*		48·64
	Magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester		1·06
	Total chlorophylls		115·37

† Isolated from saponified extracts. Present in unsaponified cells as siphonein (xanthophyll K1) and xanthophyll K2.

* The culture harvested had a cell concentration of $2{\cdot}3 \times 10^6$ cells/ml. The average cell values were as follows: volume, $5{\cdot}4 \times 10^{-11}$ ml; approximate calculated wet weight, 54 pg; approximate calculated dry weight, 6·5 pg. Chlorophylls and carotenoids constituted 1·8 and 0·5 per cent respectively of the cell dry weight.

TABLE 5. THE ABSORPTION MAXIMA, RELATIVE POLARITIES AND R_f'S OF THE KETO-XANTHOPHYLLS OF THE SCALY FLAGELLATES AND OF THEIR REDUCTION AND SAPONIFICATION PRODUCTS COMPARED WITH THOSE OF FUCOXANTHIN, SIPHONEIN AND SIPHONAXANTHIN

Xanthophyll	Isolated from	Absorption maxima (nm)§			Relative polarity	R_f on polyamide TLC†	
		Petroleum spirit (40–60°)	Ethanol*	Chloroform		System A	System B
Micronone	*M. Nephroselmis*	(416), 439·5, 467	442–7	451–5	2·07	0·44	0·74
Microxanthin (reduced micronone)			(375), 396, 419, 446	(381), 403, 427, 455	2·38	0·27	0·79
Xanthophyll K	*M. squamata*, *Nephroselmis*	(429), 452, 480	446–454	455–467	2·51	0·25	0·78
Reduced xanthophyll K			(376), 398, 421, 448	(381), 405, 429, 457	2·66‡	0·19	0·80
Xanthophyll K1	*P. amylifera*, *H. longifilis*	(428), 450, 477	454–466	467		0·34	0·72
Siphonein	*C. fragile*	(427), 450, 478	452–464	466	2·00	0·34	0·73
Xanthophyll K1S		(428), 450, 478	444–452	458–468	3·26	0·22	0·87
Siphonaxanthin	*C. fragile*	(427), 450, 478	445–451	466	3·21	0·23	0·87
Reduced xanthophyll K1			(375), 398, 421, 448	(380), 405, 429, 458		0·30	
Reduced siphonein			(375), 397, 420, 448		2·09	0·29	
Reduced xanthophyll K1S			(376), 398, 421, 448	(380), 405, 429, 456		0·19	0·85
Reduced siphonaxanthin			(375), 398, 421, 448			0·21	
Xanthophyll K2	*P. amylifera*, *H. longifilis*	(427), 449, 476	455–465	466		0·29	0·77
Xanthophyll K2S			444–454	457–467		0·16	0·90
Reduced xanthophyll K2			(375), 398, 421, 448	(380), 404, 429, 457		0·27	
Reduced xanthophyll K2S			(376), 398, 421, 448	(380), 405, 429, 456		0·14	0·89
Fucoxanthin‖	*Prymnesium parvum*	(425), 447·5, 475	447–452	454–461	2·32‖	0·28	0·95
Reduced fucoxanthin			(378), 399, 422, 448·5			0·25	
Product of saponification of fucoxanthin			(378), 399, 422, 449	(383), 406, 429, 458		0·25	
Product of saponification of fucoxanthin			450·5	461		0·18	

* None of the xanthophylls showed any change in maxima on addition of hydrochloric acid (0·005 N final concentration).
† Lutein showed R's of 0·35 and 0·68 when chromatographed in Systems A and B respectively at the same time as the other xanthophylls.
‡ From *Micromonas squamata*.[10]
§ Brackets indicate the approximate position of a point of inflexion. A range of values indicates a flat maximum.
‖ Approximate relative polarity calculated from the data of Jensen.[17] The two principal products of saponification are shown below reduced fucoxanthin.

F6X found in older cultures of *M. squamata.*[10] The results indicate the presence of a single conjugated carbonyl grouping in the molecule.

The identities of the other named carotenoids were ascertained as indicated in the Methods Section.

The pigment composition of *Pyramimonas obovata* is shown in Table 2. It much resembles those of the scaly flagellates *Platymonas chuii* and *Prasinocladus* sp.[10] All these organisms show a relatively normal chlorophycean pigment pattern.

Trace amounts of an unidentified carotene P3 were detected. This showed absorption maxima at (455),482·5 and 516 nm in petroleum spirit (b.p. 40–60°) and at (470),497 and 528 nm in chloroform. It was eluted after γ-carotene when chromatographed on alumina and was epiphasic to 95% aqueous methanol in petroleum spirit solution. Insufficient material was available for further investigation.

The xanthophyll named as ?trollein was identical with the xanthophylls of the same name present in *Prasinocladus* sp. and *Platymonas chuii.*[10] Details of its R_f on polyamide TLC are given in Table 6.

The pigment composition of *Pyramimonas amylifera* is shown in Table 3.

Two ketonic xanthophylls K1S and K2S, constituting 36·5 per cent of the total carotenoids of this flagellate, were more strongly adsorbed on icing sugar columns than neoxanthin. Details of these xanthophylls and of their products of borohydride reduction (after purification) are given in Table 5. Borohydride reduction was slow and incomplete even after 1 hr at room temperature. Xanthophyll K2S was virtually insoluble in petroleum spirit (b.p. 40–60°) and xanthophyll K1S only very slightly soluble. It was therefore impossible to carry out r.p. determinations on the former or on their reduction products. The two xanthophylls are discussed later. It will be shown that xanthophyll K1S is siphonaxanthin, derived from siphonein present in the cell by the saponification procedure. Xanthophyll K2S is a new pigment resembling siphonaxanthin and is formed from a xanthophyll resembling siphonein present in the cell by the saponification procedure.

TABLE 6. THE R_f VALUES OF XANTHOPHYLLS ON POLYAMIDE THIN-LAYER CHROMATOGRAPHY

Xanthophyll	R_f* System A	R_f* System B
Monohydroxy,mono-5,6-epoxy-α-carotene	0·76	0·76
Lutein	0·35	0·68
Zeaxanthin†	0·32	0·59
Xanthophyll B	0·31	0·61
Lutein 5,6-epoxide	0·33	0·77
Violaxanthin	0·33	0·86
? Trollein	0·16	0·76
Neoxanthin	0·25	0·87

* System A; developed with petroleum spirit (b.p. 100–120°): methanol: methyl ethyl ketone, 8:1:1 v/v/v. System B; developed with water:methanol:methyl ethyl ketone 1:5:5 v/v/v.

† Gave a partition coefficient of 35:65 between hexane (67–70°) and 85% v/v aqueous methanol and a % III/II value[12a] of 41 (cf. zeaxanthin[12b]).

[12a] S. L. JENSEN, *Kgl. Norske. Videnskabs Selskab. Skrifter* Nr. 8, 119 (1962).
[12b] D. J. CHAPMAN, *Arch. Microbiol.* **55**, 17 (1966).

The pigment composition of *Heteromastix longifilis* is shown in Table 4. It can be seen that there is a marked resemblance in carotenoid composition to that of *Pyramimonas amylifera*.

The minor unidentified xanthophyll A was a 5,6-epoxide of r.p. 1·13. It showed absorption maxima: chloroform; 446,474 nm: ethanol; (420),440,465 nm: ethanol; (377),400,422,448 nm after HCl treatment. Insufficient material was available to carry out further investigations. The results suggest a monohydroxy-5,6-monoepoxy-α-carotene structure.

The two ketonic xanthophylls K1S and K2S were identical with those of *Pyramimonas amylifera* and, like them, formed by the saponification process from siphonein and a new xanthophyll resembling siphonein respectively. Details are given in Table 5. They are discussed in the next section.

(b) Identity of the Carotenoids

This has been discussed to some extent earlier. The structures of micronone and the unidentified xanthophyll B have been discussed in Ricketts.[12] Xanthophyll B and the reduction products of xanthophylls K, K1S and K2S may be hydroxy-derivatives of 5,8-monoepoxides of bicyclic xanthophylls such as neochrome, which shows absorption maxima at (375),398,421 and 449 nm in ethanol; or hydroxy-derivatives of α-zeacarotene which shows absorption maxima at 399,421 and 449 nm in hexane.[13] The possibility also exists that the carbonyl-containing xanthophylls may be related in structure to apo-xanthophylls or to fucoxanthin or siphonaxanthin. The apo-xanthophyll sintaxanthin[14] displays absorption maxima at (425),448 and 475 nm in hexane and 462 nm in benzene; β-apo-8′-carotenal[15] has maxima at 451 and 478 nm in petroleum spirit and 463 nm in ethanol. The product of borohydride reduction showed absorption maxima at 404,425 and 451 nm in petroleum spirit in both cases. On purely taxonomic grounds it seems unlikely that the carbonyl-containing xanthophylls are apo-carotenoids as none have so far been isolated from the algae to my knowledge. The absorption maxima of the xanthophylls are very similar to those of fucoxanthin (Table 5). Moreover borohydride reduction of this purified fucoxanthin (isolated without saponification from *Prymnesium parvum* Carter) yielded products showing absorption maxima at (378),399,422 and 448·5 nm in 95 per cent aqueous ethanol (v/v) (see also Jensen[16]) and showed a single yellow spot (presumably a mixture of fucoxanthols a and b) in addition to the original fucoxanthin on polyamide TLC. Xanthophyll K is the only pigment with a relative polarity similar to that of fucoxanthin (calculated to be approximately 2·32 from the data of Jensen).[17] The R_f's of the xanthophylls are given in Table 5. It can be seen that micronone, xanthophyll K1S and xanthophyll K2S are not identical with fucoxanthin. The R_f's of xanthophyll K are similar in one system but differ in the other. Fucoxanthin and its reduction products gave a blue colour when dissolved in ether and treated with concentrated HCl (see also[16]) whereas micronone, xanthophylls K, K1S and K2S and their reduction products did not.

As fucoxanthin is known to be unstable to alkalis the possibility that the keto-xanthophylls were artifacts arising from the saponification procedure was considered. A sample of fucoxanthin was saponified in methanol containing one tenth by volume of 60% aqueous

[13] B. H. Davies, In *Chemistry and Biochemistry of Plant Pigments* (Edited by T. W. Goodwin), p. 489. Academic Press, New York (1965).
[14] H. Yokoyama and M. J. White, *J. Org. Chem.* **30**, 3994 (1965).
[15] H. Yokoyama and M. J. White, *Phytochem.* **5**, 1159 (1966).
[16] A. Jensen, *Acta Chem. Scand.* **15**, 1605 (1961).
[17] A. Jensen, *Acta Chem. Scand.* **15**, 1604 (1961).

KOH for 5 min at 40° in the dark under an atmosphere of nitrogen. The products were isolated by chromatography on magnesium oxide: Celite Hyflo Supercel 1:1 w/w, developing with petroleum spirit containing increasing amounts of ethanol. Two principal products were obtained. The least strongly adsorbed, which was yellow in solution and greenish when dry, showed absorption maxima at (378),399,422 and 449 nm in ethanol (Table 5). It gave an immediate intense blue colour when dissolved in ether and treated with concentrated HCl. The more strongly adsorbed orange product gave a more slowly developing blue colour on treatment in this way. It showed absorption maxima at 450·5 nm in ethanol (Table 5) resembling fucoxanthin but was more strongly adsorbed. It is to be noted that the less strongly adsorbed component corresponded in both R_f on polyamide TLC and in absorption maxima with the product obtained on borohydride reduction of fucoxanthin. It is possible that a molecular rearrangement has taken place resulting in the formation of a product identical to a fucoxanthol. No trace of the original fucoxanthin remained after saponification. Heilbron and Phipers[18] saponified fucoxanthin for 24 hr in 5% methanolic KOH and obtained three yellow products which showed similar absorption maxima (422,448,474 nm in carbon disulphide) which are presumably related to the yellow product obtained in the present investigation with a much shorter saponification period. J. S. Sørensen (reported in Liaaen and N. A. Sørensen[19]) found that treatment of fucoxanthin with potassium carbonate in methanol resulted in five main products. Two resembled the yellow pigment and the remainder the fucoxanthin-like pigment obtained in the present investigation. I.r. spectroscopic examination indicated that all the five pigments had lost the conjugated keto-grouping. The results obtained indicate that the keto-xanthophylls of the flagellates are not identical with fucoxanthin but may well be fairly closely related in structure to it. The only description of the presence of fucoxanthin in the green algae is that of Carter *et al.*[20] for *Zygnema pectinatum*.

The xanthophylls siphonein and siphonaxanthin, which are present in siphonalean green algae, greatly resemble fucoxanthin[21, 22, 23] but do not give a blue colour with HCl when dissolved in ether. These siphonalean green algae usually contain α- and β-carotenes, lutein, zeaxanthin, siphonein, violaxanthin, neoxanthin and siphonaxanthin in addition to chlorophylls *a* and *b*.[21] They are also noteworthy in that α-carotene predominates over β-carotene.

Siphonein and siphonaxanthin displayed single absorption maxima at about 460 and 455 nm respectively in ethanol and both showed absorption maxima at 452 and 480 nm in petroleum spirit.[22] Siphonein was adsorbed below and siphonaxanthin above neoxanthin on icing sugar columns. The former had similar adsorptive properties to fucoxanthin.[21] Siphonein was converted to siphonaxanthin upon saponification with alcoholic potassium hydroxide. It has been suggested that siphonein is an ester of siphonaxanthin.[23]

A comparison of the characteristics of siphonaxanthin with those of xanthophylls K1S and K2S indicated considerable similarities. Xanthophyll K showed similarity to siphonaxanthin in absorption maxima but had adsorptive properties reminiscent of siphonein. It

[18] I. M. HEILBRON and R. F. PHIPERS, *Biochem. J.* **29**, 1369 (1935).
[19] J. S. SØRENSEN reported in S. LIAAEN and N. A. SØRENSEN, *2nd Int. Seaweed Symp.* (Edited by T. BRAARUD and N. A. SØRENSEN), p. 25. Pergamon Press, Oxford (1956).
[20] P. W. CARTER, I. M. HEILBRON and B. LYTHGOE, *Proc. Roy. Soc. (London) Ser. B*, **128**, 82 (1939).
[21] H. H. STRAIN, *Chloroplast Pigments and Chromatographic Analysis*, 32nd Ann. Priestley Lect., p. 37. Pennsylvania State University, Penn. (1958).
[22] H. H. STRAIN, In *Manual of Phycology* (Edited by G. M. SMITH), p. 243. Chronica Botanica Co., Waltham, Mass. (1951).
[23] H. H. STRAIN, In *Photosynthesis in Plants* (Edited by J. FRANCK and W. E. LOOMIS), p. 162. Iowa State College Press, Ames (1949).

was unlikely to have been siphonein as this would not have survived conversion to siphonaxanthin during the saponification process. This comparison led to a consideration of the desirability of examination of unsaponified extracts of the flagellates.

Column chromatography of unsaponified pigment extracts of *H. longifilis* and *Pyramimonas amylifera* on icing sugar revealed two orange bands. The largest band, xanthophyll K1, adsorbed between violaxanthin and neoxanthin, showed a single absorption maximum at 454–466 nm in ethanol (Table 5). Saponification, as described earlier, resulted in almost complete conversion to the more polar orange xanthophyll K1S which showed an absorption maximum at 444–452 nm in ethanol (Table 5). The second orange band, xanthophyll K2, was adsorbed above neoxanthin and showed an absorption maximum at 455–465 nm in ethanol (Table 5). Saponification in the same way resulted in conversion to the more polar orange xanthophyll K2S, absorbing at 444–454 nm in ethanol (Table 5). Borohydride reduction of both of these saponified (K1S and K2S) and unsaponified (K1 and K2) pigments gave products with identical chromatophores, but with different R_f's on polyamide TLC in each case (Table 5). None of these pigments or reduction products gave a blue colour with concentrated HCl when dissolved in ether. The results clearly show that xanthophylls K1 and K1S are identical with siphonein and siphonaxanthin respectively of the Siphonales. Xanthophylls K2 and K2S are almost certainly related to siphonein and siphonaxanthin in structure and probably possess an additional polar group or groups. The unsaponified xanthophyll K2 is, like siphonein, converted to a more polar xanthophyll upon saponification for 5 min in 6% methanolic KOH at 40°. This change may well reflect hydrolysis of ester groupings.

These results prompted an investigation of the effect of saponification upon the micronone and xanthophyll K of *Micromonas* and *Nephroselmis*. Saponification at 40° for 30 min in 6% methanolic KOH of micronone produced no change, whereas after this treatment about 30 per cent of xanthophyll K had been converted into a product which was identical with micronone in terms of absorption spectra (both untreated and after borohydride reduction) and in R_f on polyamide TLC. These results obviously indicate that a reinvestigation of *Micromonas* and *Nephroselmis* using unsaponified pigment extracts is desirable to determine how much of the micronone recovered in saponified extracts is produced by the saponification process and whether xanthophyll K (equivalent to xanthophyll F6X in *M. squamata*) has arisen from the hydrolysis of a less polar xanthophyll in a manner analogous to the siphonein to siphonaxanthin conversion. That all the micronone isolated in saponified extracts arises from the saponification of xanthophyll K seems very unlikely for *Nephroselmis* and *M. squamata* as the 5 min saponification period employed would have resulted in only a very small percentage conversion. The results for young and older cultures of *M. squamata* may also be compared.[10] The young cultures show a high concentration of micronone whereas older cultures show a low concentration in spite of the fact that they had both received the same saponification period. It is possible however that the high concentrations of micronone reported in *M. pusilla*[12] could have arisen due to the overnight saponification period used in this instance. An investigation of this aspect of pigment composition will be the subject of a future communication.

DISCUSSION

(*a*) *Identity of the Carotenoids*

This has been discussed to some extent in the Results Section. It seems very probable that the xanthophylls K, K2 and possibly also micronone have structures which are related

to those of siphonein and siphonaxanthin. These unidentified xanthophylls differ in (at the very least) the number of polar groups present. The latter do not affect the chromatophore, which appears to consist of eight carbon to carbon double bonds and one conjugated carbonyl grouping. The basic structure of xanthophyll K may differ from that of siphonaxanthin because of its conversion to the less polar micronone upon saponification, whereas siphonaxanthin is unaffected by this treatment. Structural investigations will have to await the isolation of larger amounts of the xanthophylls.

(*b*) *Taxonomic Significance*

The flagellates possessing scaly flagella may be divided into three groups on a pigment composition basis. The first group consists of those showing the *Micromonas* type of carotenoid distribution in saponified extracts (*M. squamata* and *Nephroselmis gilva*). The second group contains those flagellates showing zeaxanthin, neoxanthin, siphonaxanthin and a related xanthophyll as the major xanthophylls in saponified extracts (*Pyramimonas amylifera* and *Heteromastix longifilis*). The final group contains those organisms displaying a relatively normal chlorophycean pigment pattern (*Prasinocladus* sp., *Platymonas chuii* and *Pyramimonas obovata*).

Micromonas squamata and *N. gilva* bear a single layer of scales upon both their flagella and bodies whereas *Pyramimonas amylifera* and *H. longifilis* have multiple layers of scales on their bodies and flagella. The final pigment group, containing *Prasinocladus* and *Platymonas*, possess multiple layers of flagellar scales but have a thecal body covering formed from coalesced scales (*Pyramimonas obovata* in this third pigment group has similar structural features to *P. amylifera*).

It would appear (at least in the organisms so far studied) that there is a gradation in characteristics from organisms bearing one flagellum and having a single layer of scales, through flagellates with multiple flagella and with multiple scaly layers on both body and flagella, to organisms with multiple layers of flagellar scales but with a body covering of coalesced scales (thecae). The pigment compositions almost parallel this gradation but there is some overlap between groups (cf. *P. amylifera* and *P. obovata*).

It seems possible that the flagellates studied could be residual representatives of a primitive green flagellate line, ranging from very simple flagellates such as *M. pusilla* with single organelles and naked flagellum, through those with a scaly covering such as *M. squamata*, to more advanced types with a normal chlorophycean pigment pattern and a relatively rigid cell covering. Whether the latter bears any relationship to the cell wall of normal green algae is not known. Lewin[24] hydrolysed the thecae of *Platymonas subcordiformis* and failed to demonstrate any glucose in the hydrolysate. Thus the thecae cannot be closely related to the cell walls of higher plants. However, Lewin[24] also pointed out that cellulose was probably absent from the cell walls of various other unicellular green algae. Thus a relationship with them cannot be ruled out because of the absence of glucose from the hydrolysate of the thecae.

The presence of siphonein in some of the scaly flagellates may indicate a fairly close relationship with the Siphonales. This xanthophyll has not so far been described in species other than members of the Siphonales. The carotenoid distribution of those flagellates possessing siphonein differs from those reported for the Siphonales[21,22] in that β-carotene predominates over α-carotene and that large amounts of zeaxanthin are present. Lutein is not present in detectable amounts. Furthermore siphonaxanthin was not detected in unsaponified extracts, but a more polar xanthophyll resembling siphonein was present in

[24] R. A. LEWIN, *J. gen. Microbiol.* **19**, 87 (1958).

appreciable quantities. It is possible that these flagellates reflect a parallel rather than a sequential type of evolutionary development from a primitive siphonalean stock.

Whether these scaly flagellates with their siphonalean-resembling pigmentation reflect any affinity between the green algal line and those groups of organisms possessing fucoxanthin will have to await further investigation and a comparison of the structures of siphonein and fucoxanthin when these are both known. Certainly a fairly close resemblance in structure appears not unlikely in view of their absorptive and adsorptive similarities. The differences in stability on alkali treatment and in colour production on treatment with hydrochloric acid in ethereal solution must obviously reflect certain differences in structure.

A consideration of the relative importance of scale-bearing flagella and pigment composition as phylogenetic markers indicates that the former is probably an indication of a general taxonomic affinity (i.e. members of the class Prasinophyceae[1–7]) whereas by means of the latter it is possible to effect a further subdivision. Strain[21,25] has examined large numbers of plants in a wide variety of classes and concluded that there is a relationship between the occurrence of particular pigment systems and the taxonomic classification of organisms. Only minor modifications occur within particular plant groupings. On a pigment basis it might therefore be considered that the three groups of scaly flagellates enumerated above (and in particular the third group showing a relatively normal chlorophycean pattern) are not very closely related to one another. Fine structural features of the flagellates[1–7] indicate that the organisms are related, at least at the class level. The pigment groupings do not correspond with the orders of the class Prasinophyceae suggested by Christensen.[8]

EXPERIMENTAL

Cultures

Heteromastix longifilis (Butch.) Rayns (Plymouth Collection No. 58), *Nephroselmis gilva* Parke and Rayns (Plymouth Collection No. 197), *Pyramimonas amylifera* Conrad (Plymouth Collection No. 246) and *P. aff. obovata* Carter (Plymouth Collection No. 280) were kindly provided by Dr. Mary Parke, of the Plymouth laboratory, as unialgal bacteria-containing cultures. These marine flagellates were grown in Erdschrieber medium at 14° with 16 hr illumination (200 lm/ft^2) and 8 hr darkness per day. The cultures were harvested by centrifugation towards the end of the logarithmic phase of growth. Harvesting, cell numbers and packed cell volume determinations were carried out as described in Ricketts[26].

Pigments

The packed cell deposits were extracted to completion in the dark with 90% v/v aqueous acetone. The pigments were easily extractable in all cases. The pigment extract was diluted with salt solution, and extracted to completion into ether. This extract was then washed twice with large volumes of water and the ethereal extract evaporated to dryness under N_2 in the dark. The dried pigment extract was dissolved in methanol and saponified at 40° for 5 min by the addition of 60% aqueous KOH (w/v) to a final concentration of 6% (w/v),[27] cooled, diluted with water and the carotenoids extracted with diethyl ether. The chlorophyll layer was retained for a while and later re-extracted in a similar manner to ensure that all the carotenoids had been extracted. The ethereal carotenoid extract was washed with water to remove traces of KOH and methanol and then evaporated to dryness. Some pigment analyses were carried out with this saponification procedure omitted so as not to destroy certain xanthophylls. The dried saponified carotenoid residue was then dissolved in a 1:1 v/v mixture of petroleum spirit (b.p. 40–60°) and 90% v/v aqueous methanol. After shaking for some time the two layers were separated and the petroleum spirit layer re-extracted with 90% aqueous methanol. The 90% methanol layer was similarly re-extracted with petroleum spirit. The petroleum spirit layers were pooled, washed several times with water to remove any methanol and then this solution, consisting mainly of carotenes, was used for further processing. The 90% aqueous methanol layers were pooled and then diluted

[25] H. H. Strain, In *Biochemistry of Chloroplasts* (Edited by T. W. Goodwin), Vol. 1, p. 387. Academic Press, New York (1966).
[26] T. R. Ricketts, *Phytochem.* **5**, 67 (1966).
[27] N. I. Krinsky and R. P. Levine, *Plant Physiol.* **39**, 680 (1964).

with salt solution. The carotenoids (principally xanthophylls) were then extracted to completion into ether. After washing with water to remove any methanol the solution was evaporated to dryness under N_2 in the dark and then separated by chromatography.

Chlorophyll assays were carried out on aliquots of the original cultures, which had been centrifuged and the cell number in the deposits determined as described in Ricketts,[26] by the method of Parsons and Strickland.[28] Magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester was determined as described in Ricketts.[9] Approximate cell dry weights were calculated as described in Ricketts.[10]

The carotenes in the petroleum spirit fraction were separated by chromatography as described in Ricketts.[29] Any xanthophylls recovered in this fraction were added to the 90% methanol fraction and processed with it. The xanthophylls were separated as described in Ricketts.[29]

All operations were carried out in a manner which reduced exposure to light and air to a minimum. The column chromatographic procedures were carried out under an atmosphere of N_2 and hastened by applying a positive pressure to the top of the column. Dried samples of the carotenoids were stored under nitrogen in the dark at $-20°$.

Purity and Identification

The carotenes which had been separated by chromatography on alumina and then, when necessary, by chromatography on magnesium oxide: Hyflo-supercel 1:1 w/w, were chromatographed on polyamide thin-layer systems[30] developing with methanol: methyl ethyl ketone: water 50:50:1 v/v/v/ and/or on Schleicher and Schüll kieselguhr paper No. 287 developing with petroleum spirit (b.p. 60–80°) (ascending system). Their R_f's were compared with those of authentic samples of known carotenes run in the same systems. The authentic samples used were those described in Ricketts[29] or carotenes which had been proved to be identical to them. The criteria used in Ricketts[29] to identify the carotenes and xanthophylls were also used in this investigation in addition to the checks on purity and identity described above.

Xanthophylls were also identified by their relative positions on column chromatography and their absorption spectra in two solvents. In addition 5,6-epoxides were detected by treating ethanolic solutions of the pigments with dilute aqueous HCl to give a final concentration of 0·005 N. All xanthophylls were tested in this way but only positive results are recorded. Polyamide thin-layer chromatography using the method of Egger and Voigt[30] and developing with petroleum spirit (b.p. 100–120°): methanol: methyl ethyl ketone 8:1:1 v/v/v (System A) or with water: methanol: methyl ethyl ketone 1:5:5 v/v/v (System B) proved a valuable aid to identification. The R_f's obtained with the xanthophylls, monohydroxy, mono-5,6-epoxy-α-carotene, lutein, zeaxanthin, unidentified xanthophyll B, lutein 5,6-epoxide, ?trollein and neoxanthin are given in Table 6. Their absorption maxima and relative polarities were in good agreement with the values published in Ricketts.[10, 12, 29]

Relative polarities were determined and borohydride reductions carried out by the methods of Krinsky.[31]

All absorption spectra were determined with an Optica Double-beam Grating Recording Spectrophotometer, Type CF4R. For the quantitative determination of the approximate carotenoid concentration of the unidentified pigments and of siphonein and siphonaxanthin $E^{1\%}_{1\,cm}=2500$ was assumed. The extinction coefficients used for the carotenes were those given in Davies[13] and those used for the xanthophylls those given in Krinsky and Levine.[27]

Fucoxanthin was isolated from unsaponified extracts of *Prymnesium parvum* Carter. Siphonein and siphonaxanthin were isolated from *Codium fragile* (kindly provided by Dr. W. Mackie, of the Astbury Department of Biophysics, The University of Leeds) without saponification of the pigment extract.

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[28] T. R. PARSONS and J. D. H. STRICKLAND, *J. Marine Res.* **21**, 155 (1963).
[29] T. R. RICKETTS, *Phytochem.* **6**, 19 (1967).
[30] K. EGGER and H. VOIGT, *Z. Pflanzenphysiol.* **53**, 64 (1965).
[31] N. I. KRINSKY, *Anal. Biochem.* **6**, 293 (1963).

Note Added in Proof

Following the original preliminary reports (Ricketts[9,12]) of the occurrence of new keto-xanthophylls in *Pyramimonas amylifera*, Riley and Wilson[32] have recently examined unsaponified acetone extracts of the flagellate by thin-layer chromatography. In addition to β-carotene, violaxanthin and neoxanthin, they found pigments which are probably identical to the siphonein and xanthophyll K2 described in the present communication. Riley and Wilson[32] also described the presence of small amounts of lutein. They did not detect zeaxanthin, which was a major xanthophyll component found in the present investigation.

An investigation of the structures of the new xanthophylls is being undertaken in collaboration with Professor B. C. L. Weedon, Queen Mary College, London.

[32] J. P. RILEY and T. R. S. WILSON, *J. Marine Biol. Assoc. U.K.* **47**, 351 (1967).