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The lipid-soluble pigments of the marine red alga *Lenormandia prolifera*

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KURZFASSUNG: **Die lipidlöslichen Farbstoffe der marinen Rotalge *Lenormandia prolifera*.** In der Rotalge *Lenormandia prolifera* (C. AG.) J. AG. ist neben den Xanthophyllen Lutein und Zeaxanthin auch das Xanthophyll α-Kryptoxanthin gefunden worden. Chlorophyll *a* ist vorhanden, nicht jedoch Chlorophyll *d*. α-Carotin tritt in größeren Mengen als β-Carotin auf, während Lutein häufiger als Zeaxanthin vorkommt. Der Gehalt an α-Kryptoxanthin beträgt nur 2,4 % der Gesamtmenge an Carotinoiden.

INTRODUCTION

Several authors have examined carotenoids in algae (see summaries in SMITH 1951, GOODWIN 1965, 1966, STRAIN 1958, 1966) but few detailed quantitative studies using thin-layer chromatography have been published. Recent workers (STRAIN 1958, 1966, CHAPMAN 1966) have shown that the only carotenoids found in the Rhodophyta are α- and β-carotene, lutein and zeaxanthin, although earlier workers had reported finding taraxanthin (HEILBRON 1942, HEILBRON, PARRY & PHIPERS 1935) and neoxanthin (ALLEN et al. 1964) in some species. ALLEN et al. (1964, p. 268) state that "the main qualitative differences observed are the presence or absence of α-carotene and the presence or absence of zeaxanthin". STRAIN (1958, 1966) has shown that in those species where α-carotene occurs, β-carotene is usually the more abundant carotene, while in some species, zeaxanthin is more abundant than lutein.

Lenormandia prolifera (C. AG.) J. AG. is a marine red alga (Ceramiales; Rhodomelaceae) common along the South-Eastern Australian coast. The genus is endemic to Australia and New Zealand and is a representative of the Amansia group, which is widely distributed in temperate and warm-temperate seas.

L. prolifera grows in rock-pools and channels in the littoral zone where overhanging rock-ledges, sand or canopies of large brown algae provide some shade.

Earlier work has shown that in *L. prolifera* both the water-soluble red pigments R-phycoerythrin and floridorubin are present (SAENGER, ROWAN & DUCKER 1968). The work reported here shows that the lipid-soluble pigments also differ from those in the majority of the Rhodophyta.

MATERIAL AND METHODS

The plants, collected at Point Lonsdale, Victoria, Australia and transported in seawater, were cleansed of epiphytes and sand and briefly rinsed in distilled water.

Extraction: The thalli were blended in 90 % acetone and the pigments extracted until the acetone was colourless. The acetone fractions were combined and partitioned with diethyl ether with the addition of 10 % NaCl solution. The ether fraction was washed with distilled water several times to remove residual acetone and was then dried over anhydrous Na_2SO_4 in a N_2 atmosphere.

Chlorophylls were removed by saponifying the solution with 30 % KOH : methanol (1:10, v/v) for 24 hours under N_2. This was partitioned with diethyl ether as described above and the saponified chlorophylls were removed in the aqueous methanolic phase.

Chromatography: The chromatography was carried out at room temperature in darkness. The chlorophyll and carotenoids were separated on plates of silica gel G (Merck, Darmstadt) developed with petroleum ether (b. p. 100–120° C) : acetone : chloroform (50:20:40, v/v). The chlorophyll was further purified using two-dimensional paper chromatography (JEFFREY 1961) on Whatman 3MM paper, previously washed with the chromatography solvents. We used *n*-propanol : petroleum ether (1:25, v/v) and chloroform : petroleum ether (3:7, v/v) as the solvents. Carotenoids were separated and purified further using plates of Al_2O_3/MgO (3:1, w/w) (CHAPMAN 1965) with ethyl acetate : *n*-hexane (3:47, v/v) as the solvent. Silica gel G/MgO (1:1, w/w) plates (CHAPMAN 1965) were used for the more strongly absorbed carotenoids, and these were developed with acetone : *n*-hexane (1:4, v/v). All Rf values are means from at least eight chromatograms. Pigments were eluted in diethyl ether, evaporated to dryness and redissolved in the solvent required for estimation.

Estimation: The $E^{1\,\%}_{1\,cm}$ values from DAVIES (1965) were used for the carotenoids and the extinction values of MACKINNEY (1940) for chlorophyll *a*. The approximate concentration of total carotenoids in saponified extracts was calculated using an $E^{1\,\%}_{1\,cm}$ value of 2500. Spectra were measured with the Beckman DB spectrophotometer using solvents of reagent grade. All results are expressed on a dry-weight basis.

RESULTS

Chlorophyll

Only chlorophyll *a* and chlorophyllide *a* were detected. The Rf values of both compounds agree closely with the values published by JEFFREY (1961). The absorption maxima in diethyl ether for chlorophyll *a* (see Table 1) are similar to the maxima published by ZSCHEILE & COMAR (1941). After repeated chromatographing on prewashed paper, the ratio of absorbance of the blue and red maxima was 1:32, indicating the absence of substantial amounts of coloured impurities (HOLT 1965).

Chlorophyllide *a* was present only in traces and apart from determining that the absorption maxima of the chlorophyllide *a* were identical with those of chlorophyll *a*

(HOLT & JACOBS 1954) we carried out no other examination on it. Since BARRETT & JEFFREY (1964) found that 80 to 100 % acetone almost completely inhibited chlorophyllase, the chlorophyllide *a* was probably not produced by degrading chlorophyll *a* during extraction.

Attention was given to the possible presence of chlorophyll *d*. No other green zones apart from the chlorophyll *a* and the chlorophyllide *a* appeared in any of the chromatograms. Overlapping of the chlorophyll *d* with any of the other two chlorophyllous compounds did not occur judging by their optical purity. Thus, chlorophyll *d* was absent in the material examined.

Carotenoids

The primary separation of the carotenoids gave three distinct zones with Rf values as follows: fraction 1 (F1) – 1.0; F2 – 0.73; F3 – 0.39. Some sub-zoning occurred in both F1 and F3, although the sub-zones could not be eluted separately. After elution, F1 was chromatographed on Al_2O_3/MgO plates and one yellow and one orange zone separated with Rf values of 0.98 and 0.80 respectively. Their Rf values and absorption maxima coincided with α- and β-carotene respectively. Co-chromatography on both silica gel G and Al_2O_3/MgO failed to separate the two compounds of F1 from authentic α- and β-carotenes from carrot root (KARRER & JUCKER 1948).

Table 1

The spectral properties and concentration of the lipid-soluble pigments present in *Lenormandia prolifera*. Figures in italics are the III/II % values published by HAGER & MEYER-BERTENRATH (1967b). Figures for absorption in brackets represent inflexions rather than peaks

Pigment	mg/g dry wt.	% Total carotenoid	Absorption maxima (mμ)			Solvent	III/II value (%)	
α-carotene	0.16	19.8	423	445	475	n-hexane	59	*65*
			432	456	485	chloroform		
β-carotene	0.12	15.1		451	481	n-hexane	24	*27*
				465	493	chloroform		
α-cryptoxanthin	0.02	2.4	423	446	475	n-hexane	68	*71*
			422	448	478	ethanol		
			432	458	488	benzene		
			420	446	477	n-hexane[1]		
			422	448	478	ethanol[1]		
Iodine isomerization mixture			(418)	439	468	n-hexane		
Neo α-cryptoxanthin A			(417)	439	469	petroleum spirit		
Lutein	0.41	50.0	423	447	477	n-hexane	57	*60*
			426	456	487	chloroform		
Zeaxanthin	0.10	12.7		451	482	n-hexane	27	*25*
				461	492	chloroform		
Chlorophyll *a*	1.41		410	432	533	diethyl ether		
			575	612	658	diethyl ether		
Total carotenoids	0.81							
Total pigment	2.22							

[1] Data of CHOLNOKY et al. (1958).

F3 separated into one yellow and one orange zone on silica gel G/MgO plates with Rf values of 0.25 and 0.08 respectively. The absorption spectra coincide with lutein and zeaxanthin respectively. Co-chromatography of the respective pigments with authentic samples of lutein and zeaxanthin, obtained from *Laurencia heteroclada* HARVEY (STRAIN 1966), failed to separate these pigments on both silica gel G and silica gel G/MgO plates.

The F2 failed to separate further on all plates used and we assumed that it was homogenous. Both the Rf values and the absorption maxima suggest that this carotenoid is α-cryptoxanthin, a mono-hydroxy derivative of α-carotene. Maxima for α-cryptoxanthin (CHOLNOKY, SZABLOCS & NAGY 1958) are given in Table 1. Epiphasic behaviour of the pigment with 90 % methanol : *n*-hexane (1:1, v/v) and a negative 5,6-epoxide-HCl test (HAGER & MEYER-BERTENRATH 1967a) support this identification. A III/II % value of 68 for the pigment compared closely with a value of 71 obtained for α-cryptoxanthin by HAGER & MEYER-BERTENRATH (1967b).

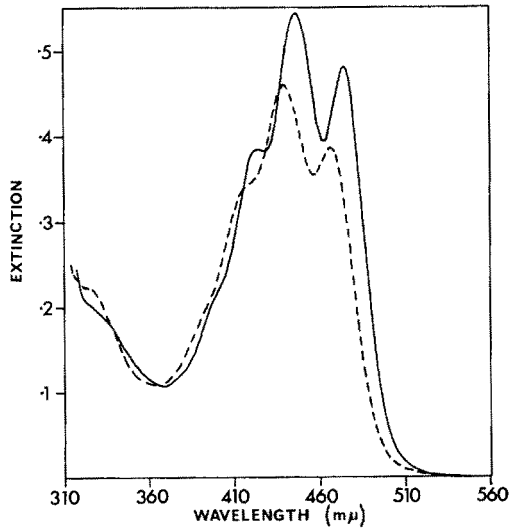

Fig. 1: The absorption spectra in n-hexane of the carotenoid identified as α-cryptoxanthin before (———) and after (– – –) iodine isomerization

The absorption in *n*-hexane of the all-trans form of the cartenoid and that of the iodine equilibrium mixture are shown in Figure 1. With iodine isomerization, the drop in extinction at λmax was 16 % while a slight cis peak was observed at 327 mμ with $E^{Mol}_{1\,cm} = 5.8 \times 10^4$, the molecular extinction falling within the range given by DAVIES (1965). The small amount of pigment available restricted further chemical examination using IR or NMR spectroscopy.

The results of quantitative analyses of all the lipid-soluble pigments in an extract from *L. prolifera* are summarized in Table 1, together with relevant spectroscopic data.

DISCUSSION

The results in Table 1 show that *L. prolifera* contains α- and β-carotene and three hydroxy derivatives of these two carotenes. Both α- and β-carotene, lutein and zeaxanthin have been reported by numerous authors and they appear to form the usual carotenoid pattern in the Rhodophyta. No violaxanthin, taraxanthin or neoxanthin was detected. α-Cryptoxanthin has not previously been reported in the Rhodophyta so far as we are aware. α-Cryptoxanthin could be present in extracts from *L. prolifera* for three reasons: (1) α-Cryptoxanthin is widely distributed in small amounts in the Rhodophyta but has been overlooked using techniques other than thin-layer chromatography: (2) α-Cryptoxanthin is an artefact derived from α-carotene during extraction. (3) α-Cryptoxanthin is restricted to a few species in the Rhodomelaceae, or even to the Amansia group. Since α-cryptoxanthin was not found in extracts from *L. heteroclada* using identical techniques, alternatives (1) and (2) seem unlikely. The distribution of this xanthophyll in algae related to *L. prolifera* requires further investigation.

The absence of chlorophyll *d* appears to be of little significance as STRAIN (1958) has shown chlorophyll *d* present in traces in one species and absent in another species of the same genus within the Rhodomelaceae.

The high α-carotene content is of interest in that STRAIN (1958, p. 64) has shown that "in a few species of several groups (Ceramiaceae, Gracilariaceae, Grateloupiaceae and Plocamiaceae) α-carotene was the principal carotenoid hydrocarbon, lutein the predominant xanthophyll". Of 20 rhodomelaceous species examined by STRAIN (1958, 1966), 14 contained no α-carotene, 5 contained a "trace" to "some" and only *Amansia dietrichiana* GRUNOW contained "much" α-carotene. Thus the Amansia group, to which *L. prolifera* belongs also, possibly constitute a small group rich in α-carotene within the Rhodomelaceae.

SEYBOLD & EGLE (1938) have shown that the ratio of chlorophyll to carotenoid in the algae varies much less within and between species than concentrations of the pigments expressed per unit dry weight. Measuring carotenoids in saponified extracts, the mean of eight determinations of the chlorophyll/carotenoid ratio in *L. prolifera* (2.5 ± 0.7, w/w) falls within the range reported in the Rhodophyta by SEYBOLD & EGLE (1938). The variation in proportion of large, colourless medullary cells within and between species of algae undoubtedly contributes much of this variation in concentration of pigments when expressed per unit dry weight, and a parameter independent of internal differentiation should prove more reliable in examining changes in pigments in clones growing in a range of environments.

SUMMARY

1. The marine red alga *Lenormandia prolifera* contains α-cryptoxanthin, a monohydroxy derivative of α-carotene, as well as α- and β-carotene, lutein and zeaxanthin.
2. Chlorophyll *a* and a trace of chlorophyllide *a* is present but no chlorophyll *d*.

3. The chlorophyll/carotenoid ratio is 2.5 ± 0.7 (w/w), falling within the range reported for other species of the Rhodophyta.
4. α-Carotene is more abundant than β-carotene while lutein is the most abundant xanthophyll; α-cryptoxanthin accounts for only 2.4 % of the total carotenoid content.
5. The possibility that the *Amansia* group is distinguished by containing α-cryptoxanthin and more α- than β-carotene is discussed.

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