CLXVII. THE ALGAE.

II. THE RELATIONSHIP BETWEEN CERTAIN ALGAL CONSTITUENTS.

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IN a former publication [Heilbron *et al.*, 1934] covering a preliminary examination of the Phaeophyceae a description was given of the isolation of a new sterol, fucosterol, $C_{29}H_{48}O$. We have now extended our investigations to members of the Chlorophyceae and Rhodophyceae in order to ascertain whether this sterol is common to all species of algae and also to determine the character of the lipid pigments. No mention of the presence of sterols in these plants is to be found in the literature and little is known of the carotenoid pigments. Willstätter and Page [1914] showed that the marine green alga *Ulva lactuca* contained only carotene and xanthophyll, and Kylin [1927] using qualitative capillary analytical methods for detection, but without actually isolating any pigments, stated that the lipochromes existent in the stated species were as shown in the following table:

	Carotene	Phyllorhodin	Xanthophyll	Phylloxanthin	Fucoxanthin
Higher plants	+	+	+	+	—
Chlorophyceae	+	+	+	+	—
Rhodophyceae	+	+	+	—	—
Phaeophyceae	+	—	+	+	+

The presumed pigments, phyllorhodin, phylloxanthin and even xanthophyll require re-identification in the light of our present knowledge of the plant lipochromes.

In our present study we have examined three freshwater Chlorophyceae, viz.: *Cladophora sauteri*, *Oedogonium* and *Nitella opaca*, and have taken *Rhodymenia palmata* as a typical member of the Rhodophyceae.

EXPERIMENTAL.

General procedure.

Cladophora sauteri (150 kg. wet weight) collected from Hickling Broad, Norfolk, was covered with methylated spirit (300 litres) and allowed to stand for 24 hours with intermittent agitation. The deep olive-green extract after concentration to one-third its volume was saponified at room temperature for 24 hours by the addition of 20 % aqueous KOH (2 litres). The whole was diluted with water, exhaustively extracted with ether, the ethereal solution washed with water and dried over sodium sulphate. In the case of *Rhodymenia palmata* (50 kg. wet weight) (collected from the north coast of Scotland), the plant was washed free from mucilages with 40 % acetone [see Heilbron and Phipers, 1935] and extracted with methylated spirit (50 litres), the alcoholic extract being saponified and the unsaponifiable material transferred to ether as described above.

The sterols were obtained from the non-saponifiable fractions by removing the solvent, dissolving the residues in the minimum quantity of boiling methyl alcohol and cooling to -75° .

The unsaponifiable fractions of *Nitella opaca* and *Oedogonium* were both obtained from the Torrey Research Station, Aberdeen, the sterols being isolated by us by crystallisation from methyl alcohol as above.

STEROLS AND HENTRIACONTANE.

(a) *Cladophora sauteri*.

The pale yellow semi-crystalline mass was repeatedly crystallised from methyl alcohol giving (i) a small amount of a sparingly soluble waxy material which after several recrystallisations from ethyl acetate melted at $67-68^{\circ}$ and was shown to be the paraffin hydrocarbon, hentriacontane, previously recognised as a constituent of the Phaeophyceae by Heilbron *et al.* [1934] and (ii) a more soluble portion, obviously sterolic, m.p. $80-120^{\circ}$. This latter was acetylated in the usual manner and a portion of the acetylated product (3 g.) in ether (30 ml.) treated with a 5 % solution of bromine in glacial acetic acid (40 ml.). After standing in the ice-chest for 24 hours the precipitated white powder, m.p. 130° , was directly debrominated by the method of Windaus and Hauth [1906], yielding after recrystallisation an acetate, m.p. $118-119^{\circ}$, which gave no depression in melting-point on admixture with an authentic sample of fucosteryl acetate. Hydrolysis of the acetate furnished fucosterol, m.p. 124° . After the removal of the fucosteryl acetate tetrabromide the mother-liquors were precipitated with methyl alcohol and the ether removed in a current of air. Crystallisation of the deposited material from methyl alcohol yielded a bromide, m.p. 120° (sitosteryl acetate dibromide melts at 120° [Anderson *et al.*, 1926]), debromination of which gave an acetate, m.p. 135° , $[\alpha]_D^{20} - 45.12^{\circ}$ ($c=8$ in chloroform), which on hydrolysis furnished the free sterol, m.p. 136° . (Found: C, 84.1; H, 11.8 %. $C_{29}H_{50}O$ requires C, 84.05; H, 11.9 %.) The benzoate, prepared in pyridine with benzoyl chloride, melted at 143° .

The confused state of the sitosterol literature [compare Anderson *et al.*, 1926; Bonstedt, 1928] makes it difficult to assign a specific name to our sitosterol but from the appended table it would appear that it is a mixture of the β - and γ -forms.

	Sterol M.P.	Acetate M.P.	Benzoate M.P.	Rotation of acetate
α -Sitosterol	135-136°	127-128°	145°	-17.2°
β -Sitosterol	139-140	127-128	145	-39.0
γ -Sitosterol	142	143	—	-46.1
Algal sitosterol	136	135	143	-45.1

(b) *Nitella opaca*.

The crude mass was taken up in hot light petroleum, leaving a small amount (5 mg.) of an insoluble material, m.p. 280° , which was purified by crystallisation from aqueous pyridine, giving a micro-crystalline powder, m.p. $283-285^{\circ}$. (Found: C, 72.4; H, 10.3 %. $C_{35}H_{66}O_6$ requires C, 72.7; H, 10.5 %.) This compound responds to both the Liebermann-Burchard and the Salkowski tests, and this fact, taken in conjunction with the analytical data, indicates that it is a phytosterolin, a class of compound originally discovered by Power and Salway [1913] and more recently examined by Heyl and Larsen [1934].

The light petroleum solution was evaporated and the residue crystallised from methyl alcohol, giving a small amount of a sparingly soluble low-melting residue which on recrystallisation from ethyl acetate yielded hentriacontane,

M.P. 67–68°. The crude sterols obtained on concentration of the methyl alcoholic mother-liquors were separated by bromination, and fucosteryl acetate tetrabromide, M.P. 131–133°, and sitosteryl acetate dibromide, M.P. 120°, were thus obtained.

(c) *Oedogonium*.

Similar treatment of the crude material yielded a small amount (2 mg.) of the same phyosterolin, M.P. 283–285°, as obtained above; the sterol fraction on crystallisation from methyl alcohol yielded sitosterol, M.P. 135°; no trace of fucosterol could be found but hentriacontane was again detected.

(d) *Rhodymenia palmata*.

The crude sterols, M.P. 114–115°, on repeated crystallisation from methyl alcohol, furnished hentriacontane and fucosterol, M.P. 122–123°. Bromination of a portion of the crude sterol acetate gave only fucosteryl acetate tetrabromide, M.P. 130–132°.

CAROTENOID PIGMENTS.

The deep crimson methyl alcoholic solutions obtained after removal of the sterols in the manner described were subjected to the Kraus partition with 90 % methyl alcohol and light petroleum.

Cladophora sauteri.

(a) *The light petroleum phase*. Adsorption on activated alumina and development of the chromatogram with light petroleum gave three distinct orange-red bands. The uppermost band, on elution with methyl alcohol and subsequent crystallisation from the same solvent yielded a deep red solid, M.P. 175–177° (uncorr.), having absorption maxima in carbon disulphide at 505 and 473 $m\mu$ (see later).

Attempts to crystallise the concentrated methyl alcoholic eluate of the second zone were abortive owing to the presence of terpenes and sterols. We have now found that lipochromes can be completely freed from terpenes by adsorption on fuller's earth. Whereas the pigments are strongly adsorbed, prolonged washing of the developed chromatogram with benzene effectively removes all terpene. The eluted pigment solution, now only contaminated with sterol, was further purified by adsorption on calcium carbonate; in this case the pigment passed down the column, the sterol being retained in the upper section. The pigment obtained by concentration of the final methyl alcoholic eluate yielded, on crystallisation from the same solvent, lutein as a fine red powder, M.P. 187° (uncorr.), having absorption maxima in carbon disulphide at 512 and 479 $m\mu$.

The third band passed rapidly through the column and was collected in the filtrate. Removal of solvent gave a deep red odoriferous oil from which terpenes were removed by the process described above. Elution of the pigment and repeated crystallisation from methyl alcohol-benzene furnished β -carotene in small glistening coppery plates, M.P. 178–179° (uncorr.), having absorption maxima in carbon disulphide at 518, 483 and 450 $m\mu$.

(b) *The methyl alcoholic phase*. The solvent was removed and the residue dissolved in a mixture of benzene-light petroleum (1:3) and adsorbed on activated alumina, giving a very complex chromatogram consisting of four orange-red zones. Since the eluates of the zones were spectroscopically identical (maxima in carbon disulphide at 505, 474 and 446 $m\mu$) the complexity of the chromatogram is definitely due to sterol and terpene contaminants. Concentration of the eluates yielded red solids, M.P. 175–178° (uncorr.), which were

combined with the pigment showing similar absorption maxima from the light petroleum chromatogram. Kuhn and Lederer [1932] have obtained a pigment showing the same absorption maxima from *Leontodon autumnalis*; by repeated chromatographic treatment on calcium carbonate these authors finally resolved it into taraxanthin and lutein. Adopting a similar procedure the above pigment, M.P. 175–178°, was resolved from benzene-light petroleum (1:3) by adsorption first on calcium carbonate and finally on activated alumina, taraxanthin and a further quantity of lutein being thus obtained. Crystallisation of the taraxanthin from methyl alcohol containing a trace of ether yielded glistening deep orange-red needles, M.P. 178–179° (uncorr.), having absorption maxima in carbon disulphide at 502, 472 and 442 $m\mu$. Analytical values for the mixed pigment (C, 83.1; H, 9.8 %) indicate that the ratio of lutein to taraxanthin is approximately 7:3.

Nitella opaca.

(a) *Light petroleum phase.* The solution, after adsorption and development on calcium carbonate, showed two distinct red zones, the lower and more intense of which on elution and crystallisation from benzene-methyl alcohol furnished β -carotene, whilst the upper and smaller band was found to be spectroscopically identical with lutein.

(b) *Methyl alcoholic phase.* The deep red residue obtained on removal of solvent was dissolved in a mixture of light petroleum-benzene (2:1) and adsorbed on activated alumina. Development of the chromatogram gave two broad orange-red bands, the lower of which on elution and crystallisation from benzene-methyl alcohol furnished lutein in glistening deep red plates, M.P. 189–190° (uncorr.) having absorption maxima in carbon disulphide at 511, 479 and 445 $m\mu$. The upper zone contained only a small amount of pigment, M.P. 175–176°, spectroscopically identical with taraxanthin.

Oedogonium.

(a) *Light petroleum phase.* The solution after adsorption on calcium carbonate gave a chromatogram showing three distinct bands, the upper of which, as shown by Kraus partition, consisted of traces of xanthophylls which had escaped partition. The second and main band furnished β -carotene, while the third, which was of a bright yellow colour, was spectroscopically characterised as α -carotene (absorption maxima in carbon disulphide at 510 and 478 $m\mu$).

(b) *Methyl alcoholic phase.* The pigments were adsorbed on activated alumina as previously described, when two distinct bands were obtained, the upper of which yielded taraxanthin; the lower band contained a pigment, M.P. 175–177°, which after re-adsorption on calcium carbonate was resolved into lutein and taraxanthin.

Rhodymenia palmata.

(a) *Light petroleum phase.* The lipochromes, which were resolved in the usual manner, consisted of β -carotene and a trace of lutein.

(b) *Methyl alcoholic phase.* Chromatographic analysis yielded results identical with those obtained from the corresponding phase derived from *Cladophora sauteri*, the pigments thus being taraxanthin and lutein.

DISCUSSION.

A review of the relationship between algal species and their lipid contents reveals many striking features as shown in Table I. It will be observed that each group of algae possesses characteristic lipochromes, the Chlorophyceae and

Table I.

		Lipochromes					Sterols		
		Hentriacontane	β -Carotene	α -Carotene	Lutein	Taraxanthin	Fuco-xanthin	Sitosterol	Fuco-sterol
Land plants		+	+	+	+	+	—	+	—
Chlorophyceae	<i>Oedogonium</i>	+	+	+	+	+	—	+	—
	<i>Cladophora sauteri</i>	+	+	—	+	+	—	+	+
	<i>Nitella opaca</i>	+	+	—	+	+	—	+	+
Rhodophyceae	<i>Rhodymenia palmata</i>	+	+	—	+	+	—	—	+
Phaeophyceae	<i>Pelvetia canaliculata</i> *	+	+	—	—	—	+	—	+
	<i>Fucus vesiculosus</i>	+	+	—	—	—	+	—	+
	<i>Laminaria digitata</i> *	+	+	—	—	—	+	—	+

* Unpublished investigations.

Rhodophyceae containing taraxanthin and lutein in contrast to the Phaeophyceae which are distinguished by the presence of fucoxanthin [Heilbron and Phipers, 1935]. A gradual transition from the constituents of the normal land plants is observed through the species, the Phaeophyceae bearing little resemblance to the former.

According to Fritsch [1935] the botanical properties indicate that the Oedogoniales "are a sharply circumscribed group, showing but few points of affinity with the other Chlorophyceae". Our investigations provide strong proof of this statement, and indeed as far as the chemical composition of the lipoids is concerned, these plants can be considered as land plants. The normal Chlorophyceae contain both the phytosterol, sitosterol and the algal sterol, fucoesterol. From the Rhodophyceae, sitosterol is absent, only fucoesterol being observed; although this group does not contain the land plants' sterol, it still retains the typical phyto-xanthophylls, *viz.* taraxanthin and lutein. In the Phaeophyceae even this last relationship with the land plants is severed, the characteristic pigment fucoxanthin being substituted.

Turning now to the results reported by Kylin [1927] and comparing them with our findings (see Table I) the identity of phyllo-rhodin with taraxanthin and of xanthophyll with lutein is evident. We are extremely doubtful as to the existence of a lipochrome, phylloxanthin, which Kylin states gives a green colour with 25 % hydrochloric acid, for we have repeatedly observed such a phenomenon with crude algal pigment solutions. Careful resolution, however, invariably yielded pigments giving no such colour on treatment with acid (see also preceding paper).

Reference has also been made [Heilbron *et al.*, 1934] to the interesting relationship existing between fucoesterol and the isomeric sterol of the molluscs, ostreasterol (a dehydrositosterol) [Bergmann, 1934]. The evidence now brought to light that many species of algae also contain sitosterol further emphasises the very close biological relationship between these three sterols:

SUMMARY.

The unsaponifiable material from *Cladophora sauteri*, *Nitella opaca*, *Oedogonium* and *Rhodymenia palmata* has been examined. A clear-cut change in the character of the lipoids is observed when passing from one species to another.

(1) Whereas *Oedogonium* resembles the land plants in containing only sitosterol, *Cladophora sauteri* and *Nitella opaca* produce both the former sterol and fucosterol, the latter alone being present in the marine algae *Rhodymenia palmata* and *Fucus vesiculosus*.

(2) All algae contain small amounts of the paraffin hydrocarbon hentriacontane.

(3) A new phytosterolin, m.p. 283–285°, has been isolated from *Nitella opaca* and *Oedogonium*.

(4) The lipochrome pigments lutein, taraxanthin and β -carotene were isolated from all species and α -carotene was observed in *Oedogonium*. This presents a marked contrast to the Phaeophyceae (see preceding paper).

The evidence adduced shows that the Oedogoniales show a closer chemical relationship to the land plants than to the typical algae.

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