CLXVI. THE ALGAE.

I. THE LIPOCHROMES OF *FUCUS VESICULOSUS*.

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THE earliest recorded investigation of the fat-soluble pigments of the brown algae was made by Cohn [cited by Kylin, 1912], who isolated from the Phaeophyceae one brown pigment, phaeophyll, which he presumed was closely related to chlorophyll. Millardet [1869] clarified the position by showing that phaeophyll could be resolved by partition processes between light petroleum and alcohol into chlorophyll, a yellow lipochrome, phycoxanthin, and a brown water-soluble pigment, phycophain. Sorby [1873] came to the conclusion that the Fucaceae, a branch of the Phaeophyceae, contained three different yellow lipochrome pigments, xanthophyll, phycoxanthin (which he renamed fucoxanthin) and lichnoxanthin, the last occurring in very small amounts. Reinke [1876] considered that Sorby's method of distinguishing between the yellow pigments was unreliable and was himself only able to detect fucoxanthin, which was not identical with the xanthophyll of the higher plants. Tswett [1906], employing the chromatographic method, confirmed the presence of carotene first demonstrated by Tammes [1900] and also of fucoxanthin, the presence of which had recently been denied by Gaidukow [1903]. Tswett also distinguished a further lipochrome pigment, fucoxanthophyll, and showed that whereas fucoxanthin develops an intense royal blue colour with hydrochloric acid, fucoxanthophyll gives only a green colour. Kylin [1912] further defined the colour reactions of fucoxanthin and stated that fucoxanthophyll was identical with the xanthophyll of the higher plants (which gives no colour with hydrochloric acid). Willstätter and Page [1914] isolated fucoxanthin as a crystalline compound from various Phaeophyceae and assigned to it the formula $C_{40}H_{54}O_6$ or $C_{40}H_{56}O_6$. By partition processes between aqueous methyl alcohol, ether and light petroleum, they further obtained a pigmented solution which gave no colour with hydrochloric acid and on these grounds concluded that "xanthophyll" was also present. These authors observed that it was apparently impossible to obtain fucoxanthin from brown algae after a period of 10 days had elapsed from the time of gathering; they did not, however, pursue this matter further.

In view of the wide disparity in the results of the earlier investigators we have again carried out an examination of the lipochromes present in *Fucus vesiculosus* and have employed for this purpose both the fresh alga and old dried material. Our results show that whereas fresh *Fucus vesiculosus* contains the characteristic brown algal pigment fucoxanthin together with carotene, the former pigment is wholly absent from the dried material, which contains in its place zeaxanthin. In addition a constitution for fucoxanthin based on its chemical properties, and permitting of such a post-mortal transformation, is discussed later in the paper.

EXPERIMENTAL.

Old dry *Fucus vesiculosus*. The material, collected during the summer months from the north coast of Scotland, was air-dried over a prolonged period and milled, giving a pale fawn powder which was subjected to a systematic solvent extraction, a typical experiment being outlined below [see also Heilbron *et al.*, 1934]. The powder (36 kg.) was intermittently stirred with 95 % ethyl alcohol (40 l.) for 18–24 hours at room temperature. The deep olive-green solution obtained by filtration through an edge filter was diluted with water (10 l.) and shaken with successive portions of light petroleum (B.P. 40–60°) until the extracts were practically colourless. The latter were combined and concentrated under nitrogen (extract A). The alcoholic phase, after further dilution with an equal volume of water, was completely extracted with ether, the ethereal solution washed with water, dried and concentrated to about 2000 ml. (extract C). The residual meal was extracted with ether (40 l.), the ethereal solution furnishing extract B.

Extract C. This was dried by distillation with an equal volume of benzene (final volume 400 ml.) and the cold benzene solution adsorbed in four portions on columns (50 × 5 cm.) filled with Merck standardised Brockmann alumina (1 part) and ordinary alumina (1 part), the chromatogram being developed with light petroleum (B.P. 40–60°); three distinct bands were thus obtained.

The uppermost band (deep olive-green), which could not be completely eluted with methyl alcohol, was desorbed slowly with a mixture of water, methyl alcohol and pyridine. The pigment, obviously of the chlorophyll type (probably allomerised chlorophyll), was not further examined.

Elution of the second band (deep brick-red, immediately below the green band) was performed with methyl alcohol; the solution was concentrated to small bulk, poured into water and extracted with ether. The washed and dried ethereal solution was concentrated (2 ml.) and diluted with light petroleum. After standing in the ice-chest for 24 hours, the precipitated pigment was repeatedly crystallised from benzene-methyl alcohol, from which it separated in deep red glistening needles, M.P. 211° (uncorr.). It was characterised as zeaxanthin by its absorption maxima in CS₂ (518, 483 and 451 mμ) and by analysis. (Found: C, 83.2; H, 10.1 %. C₄₀H₅₆O₂, 0.5 CH₃OH requires C, 83.2; H, 10.2 %) [cf. Kuhn and Grundmann, 1934].

The third pigmented section, formed at the base of the column, after elution with methyl alcohol and removal of solvent yielded a pale yellow mobile oil from which no crystalline pigment could be obtained. This zone consisted of terpenoid substances previously reported by Heilbron *et al.* [1934].

Extracts A and B. After removal of solvent the residue in each case was saponified with 10 % methyl alcoholic potash (3 l.) at 40° for 3 hours. The unsaponifiable material obtained by ethereal extraction was freed from fucosterol and hentriacontane by crystallisation from methyl alcohol as described by Heilbron *et al.* [1934], and the deep red methyl alcoholic mother-liquors were steam-distilled in an atmosphere of nitrogen to remove volatile terpenes. The residue was extracted with benzene and the dried concentrated solution (50 ml.) chromatographically resolved as described above. The completed chromatogram showed three distinct red bands, the uppermost of which on elution with methyl alcohol yielded a further quantity of zeaxanthin, M.P. 208–209°. The central band was also eluted with methyl alcohol and the solution concentrated until turbid. The pigment which separated on cooling was recrystallised from benzene-methyl alcohol, giving beautiful metallic flakes,

M.P. 178–179° (uncorr.), of β -carotene; absorption maxima in CS_2 , 518, 483 and 451 $m\mu$. (Found: C, 89.3, H, 10.8 %. $\text{C}_{40}\text{H}_{56}$ requires C, 89.5; H, 10.5 %.)

Elution of the brownish red zone at the base of the column provided a further quantity of terpenoid material.

Fresh Fucus vesiculosus. The minced alga (50 kg.) was freed from mucilages by washing twice with aqueous acetone (50 l. of 40 %) as described by Willstätter and Page [1914]. The washed material was covered with methylated spirit (50 l.) and allowed to stand for 24 hours with intermittent agitation. The alcoholic extract was resolved as described above into extracts A, B and C.

Extract C. This was dried with benzene and the solution (3 l.) chromatographically resolved in 15 portions in columns (50 $\times$ 5 cm.) filled with partially deactivated alumina (prepared by washing Brockmann activated alumina with methyl alcohol and subsequent drying in air) the chromatograms being developed with benzene-light petroleum (1:1) and finally washed with light petroleum (B.P. 40–60°)¹. The completed chromatogram formed three definite bands, the uppermost of which, a deep olive-green, appeared to be identical with that obtained from extract C of the old alga. The middle broad crimson band was eluted with methyl alcohol and the deep red solution diluted with an equal volume of water and allowed to stand, atmospheric oxygen being excluded by a thin layer of light petroleum. The pigment which slowly separated as a semi-crystalline precipitate at the interphase was dried *in vacuo* over sodium sulphate. On recrystallisation from ether-light petroleum pure fucoxanthin separated in deep orange-red needle aggregates, M.P. 166–168° (uncorr.); absorption maxima in carbon disulphide, 510, 477 and 445 $m\mu$; in chloroform 487 and 456 $m\mu$. (Found: C, 75.3; H, 9.3 %.) Karrer *et al.* [1931] give the M.P. as 160° (corr.) and maxima in chloroform at 492 and 457 $m\mu$. Willstätter and Page [1914] record a M.P. at 159.5–160.5° (corr.) and 158° (uncorr.). In agreement with these latter authors we have observed that whereas the blue colour produced by the action of acids on fucoxanthin exists in aqueous solution only in the presence of ether, when a light petroleum solution is treated with 25 % hydrochloric acid the blue colour remains entirely in the upper layer.

The third band on elution with methyl alcohol gave an orange-red solution, which in contrast to that obtained above, yielded its contained pigment to light petroleum on dilution. When a test portion of this was treated with 25 % hydrochloric acid a magenta colour was observed in the acid layer in addition to the royal blue colour in the light petroleum. The solution, obviously complex, was ultimately resolved by adsorption on fuller's earth, two distinct pigments being obtained. That occurring in the larger proportion on recrystallisation from ether-light petroleum yielded fucoxanthin, whilst the other (*circa* 2 mg.), which was extremely resinous, has not as yet been obtained in a crystalline state. Its ethereal solution gives with 25 % hydrochloric acid a colourless upper and a magenta lower layer and in carbon disulphide shows general absorption with a marked inflection at 500 $m\mu$. The pigment is presumably the "fucoxanthin- β " referred to but not isolated by Kylin [1927], who stated that there was a small amount of another pigment giving a more violet colour with hydrochloric acid.

We are inclined to believe that it is partially oxidised fucoxanthin, a view supported by catalytically hastening the autoxidation [*cf.* Palmer and Thrun, 1916] of the pure pigment. A solution of fucoxanthin (25 mg.) in glacial acetic acid (10 ml.) was heated on a steam-bath for 1 hour with ferric chloride (10 mg.) and the cooled solution rendered alkaline with dilute sodium hydroxide solution.

¹ If fully activated alumina is used the pigments are so strongly adsorbed that separation of the zones is impossible.

The pigment was removed with ether and the washed and dried ethereal solution evaporated. The residue was dissolved in benzene (25 ml.) and chromatographically examined on a column of activated alumina, when only one strongly adsorbed red band was obtained. Elution with methyl alcohol yielded a red solution from which no crystallisable material could be obtained. The pigment contained in this eluate was, however, identical with "fucoxanthin- β " in that treatment of an ethereal solution with 25 % hydrochloric acid gave a magenta-coloured acid layer and in CS_2 it showed a pronounced inflection at $502m\mu$.

Extract A. This was examined in detail in order to ascertain whether any "xanthophyll" other than fucoxanthin was present. The concentrated light petroleum solution (3000 ml.) was directly resolved by adsorption in 12 portions on partially deactivated alumina, giving a complex chromatogram showing five distinct bands (Fig. 1).

Fig. 1.

Band B. This band was eluted with methyl alcohol and the eluate diluted to 75 % with water and extracted twice with light petroleum. The lower phase, after separation and further dilution, yielded precipitated fucoxanthin, M.P. $161-163^\circ$. The washed light petroleum phase was dried and a test portion evaporated, dissolved in ether and treated with 25 % hydrochloric acid; a green lower layer (finally changing to blue) was thus obtained (agreement with Tswett's [1906] fucoxanthophyll). This solution should, however, according to Willstätter and Page [1914] contain "xanthophyll". The total light petroleum solution was consequently evaporated and the residue treated with cold methyl alcohol; a quantity of brown pectinoid material (*circa* 2 g.) was precipitated¹. The mother-liquors were evaporated, the residue was dissolved in the smallest volume of ether and a large excess of light petroleum added. After standing for 24 hours, the practically colourless light petroleum solution (which was found to contain fucoesterol, M.P. 124°) was decanted from the deep red crust, which, on recrystallisation from ether-light petroleum, yielded pure fucoxanthin, M.P. $166-167^\circ$ (uncorr.). It is thus evident that the green "acid" colour is not due to any specific pigment such as fucoxanthophyll but must be mainly fucoxanthin contaminated with the above materials.

¹ When using alga gathered in the winter months we have found that the amount of this substance is so large as to render the isolation of any pigment exceptionally difficult.

Band C. The bright red band on elution and resolution by the methods just described furnished fucoxanthin and fucoesterol.

Using the methods described the amounts of fucoxanthin and zeaxanthin were 1-1.5 g. and 2 g. respectively.

Fucoxanthin.

Although the analytical values clearly reveal the presence of six oxygen atoms, Zerewitinow determinations on the pure pigment indicate that only four of these exist as hydroxy-groups. The following determinations were kindly performed for us by Drs Roth and Möller of Heidelberg:

6.74 mg. fucoxanthin 0.93 ml. CH_4 (N.T.P.) : 3.9 active H.
 6.478 mg. „ 0.92 ml. „ 4.0 „

Karrer *et al.* [1931] have stated that fucoxanthin was optically active ($[\alpha]_{\text{cd}} + 73.5^\circ$ in chloroform) but a re-investigation of our higher-melting pigments shows that it is inactive ($\alpha_{\text{cd}}^\circ \pm 0.02^\circ$ in benzene) ($c = 0.062$; $l = 1$). This finding presents a striking parallel with the results obtained by Kuhn and Grundmann [1934], who by purification processes raised the melting-point of zeaxanthin from 207° to 215° and likewise found that the reported optical activity ($[\alpha]_{\text{cd}} - 70^\circ$) of this pigment was non-existent.

Repeated attempts have been made, without avail, to demonstrate the presence of carbonyl groups in the pigment, and thus, if such exist, they cannot

occupy positions analogous to those in astacene [Karrer and Loewe, 1934] or rhodoxanthin [Kuhn and Brockmann, 1933]. This does not, however, preclude the possibilities of such groups actually being present, for β -carotenone (I) [Kuhn and Brockmann, 1932] only yields a dioxime, and capsorubin (II) which according to Zechmeister and v. Cholnoky [1935] is a dihydroxyketone fails altogether to form any ketonic derivatives.

The parallel with the inert oxygen atoms in fucoxanthin leads us tentatively to suggest the possibility that this pigment has the structure (IV) (being in effect a dihydroxy-capsorubin¹). Such a constitution necessitates a formula $C_{40}H_{80}O_6$ in place of the hitherto assumed $C_{40}H_{56}O_6$, and that such an alteration is justified is seen from the following analyses.

	Carbon %	Hydrogen %
$C_{40}H_{56}O_6$	75.9	8.9
$C_{40}H_{80}O_6$	75.4	9.4
Found	75.3	9.3
Found [Karrer <i>et al.</i> , 1931]	75.5	9.1

Further, as has already been shown, the structure for fucoxanthin must be such as to accommodate a facile *post mortem* transformation into zeaxanthin (III). Whereas our suggested formula would permit of such a transformation it would be extremely difficult to account for this if fucoxanthin had hydroxy-groups in either positions $C_4:C_4'$ or $C_6:C_6'$.

Additional support of (IV) has been unexpectedly obtained by drastic perhydrogenation with an excess of platinum catalyst. The hydrogenation was carried out in acetic acid under an average pressure of 30 lbs./sq. in. The fully hydrogenated product (tetranitromethane test) crystallised from methyl alcohol-ethyl acetate in small plates, m.p. 63–64°, and was proved by analysis to have an oxygen content lower than that of the anticipated perhydrofucoxanthin. (Found: C, 82.3; H, 13.9 %. $C_{40}H_{78}O_2$ requires C, 81.4; H, 13.2 %.) Unfortunately, owing to lack of material we have so far been unable to confirm the above result, but our experiments will be continued as soon as suitable fresh *Fucus vesiculosus* becomes available.

Against formula (IV) Karrer *et al.* [1931] found that only 10 molecules of hydrogen were required to saturate fucoxanthin whereas 13 molecules would be required for a complete conversion into perhydrozeaxanthin. Further, as a product of energetic oxidation these authors only detected $\alpha\alpha'$ -dimethylmalonic acid whereas $\alpha\alpha'$ -dimethylsuccinic acid is also to be anticipated. We intend to reinvestigate these results on our chromatographically purified pigment in due course.

The fact that alcoholic alkali reacts with fucoxanthin to produce a substance much more susceptible to the action of acids was first observed by Kylin [1912] and confirmed by Willstätter and Page [1914]. We have now examined this effect in detail. Fucoxanthin (100 mg.) was allowed to stand for 24 hours with 5 % methyl alcoholic potash (25 ml.) in an atmosphere of nitrogen. The alkaline solution, after dilution, was extracted with ether. The washed and dried solution was evaporated to dryness under nitrogen and the residue dissolved in methyl alcohol. The resultant solution is extraordinarily sensitive to acids, rapidly developing a deep blue colour when exposed to the atmosphere, and this extreme lability has so far precluded attempts at recrystallisation. That the

¹ In this connection the recent isolation by Lederer [1934] of capsorubin from the ascidian *Botryllus Schlosseri* Pallas is highly significant.

products formed (which for convenience we propose to name generically the *isofucoxanthins*) are multiple is evidenced by chromatographic resolution from a benzene solution on alumina, when three distinct crimson bands are formed. It is possible to obtain a partial distinction between these components by the effect of *N*/10 acetic acid (acid of this strength being without action on fucoxanthin itself).

Band	Action of <i>N</i> /10 acetic acid	Absorption maxima in CS ₂ <i>mμ</i>	Estimated amount %
A	Slight browning	474, 448, 422	25
B	Intense royal blue	474, 448, 422	60
C	Blue colour slowly develops	475, 449, 420	15

The shift in absorption maxima implies that the number of conjugated double bonds has decreased, and bearing in mind the nature of the reaction it seems possible to us that fucoxanthin (IV) undergoes an aldol condensation, giving rise to (V).

SUMMARY.

Fucus vesiculosus has been exhaustively examined with regard to its lipochromes, and it has been found that whereas the dead material contains β -carotene and zeaxanthin the living plant contains β -carotene and fucoxanthin. These results explain the apparently contradictory statements of the earlier investigators.

No trace of the xanthophylls usually associated with the higher plants could be detected in the fresh alga, and thus zeaxanthin must be a *post mortem* product of fucoxanthin.

A chemical investigation of fucoxanthin shows that the formula of this pigment is probably C₄₀H₆₀O₆. The melting point has been raised to 166–168° (uncorr.); the pure pigment, contrary to earlier statements, is optically inactive.

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REFERENCES.

- Gaidukow (1903). *Ber. deutsch. bot. Ges.* **21**, 535.
 Heilbron, Phipers and Wright (1934). *J. Chem. Soc.* 1572.
 Karrer, Helfenstein, Wehrli, Pieper and Morf (1931). *Helv. Chim. Acta*, **14**, 614.
 ——— and Loewe (1934). *Helv. Chim. Acta*, **17**, 745.
 Kuhn and Brockmann (1932). *Ber. deutsch. chem. Ges.* **65**, 894.
 ——— (1933). *Ber. deutsch. chem. Ges.* **66**, 828.
 ——— and Grundmann (1934). *Ber. deutsch. chem. Ges.* **67**, 596.
 Kylin (1912). *Z. physiol. Chem.* **82**, 221.
 ——— (1927). *Z. physiol. Chem.* **166**, 39.
 Lederer (1934). *Compt. Rend. Soc. Biol.* **177**, 1086.
 Millardet (1869). *Ann. Sci. Nat. Bot.* **10**, 59.
 Palmer and Thrun (1916). *J. Ind. Eng. Chem.* **8**, 614.
 Reinke (1876). *Jahr. wiss. Bot.* **10**, 400.
 Sorby (1873). *Proc. Roy. Soc. Lond.* **21**, 474.
 Tammes (1900). *Flora*, **87**, 240.
 Tswett (1906). *Ber. deutsch. bot. Ges.* **24**, 236.
 Willstätter and Page (1914). *Liebig's Ann.* **404**, 237.
 Zechmeister and v. Cholnoky (1935). *Liebig's Ann.* **516**, 30.