145. *Fucoidin. Part I. The Isolation and Purification of Fucoidin from Brown Seaweeds.*

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The isolation of the polysaccharide sulphate, fucoidin, from various species of *Phæophyceæ* is described and the results of quantitative analyses are reported.

Fucoidin, the principal polysaccharide sulphate ester of the *Phæophyceæ*, differs from that of the *Rhodophyceæ* in that the main carbohydrate skeleton is founded on fucose instead of on galactose.

Fucoidin which occurs together with mannitol, alginic acid and laminarin, was first described and named by Kylin (*Z. physiol. Chem.*, 1913, **83**, 171; 1915, **94**, 357) who isolated the substance from various species of *Laminaria* and *Fucus* by extraction with dilute acetic acid and subsequent purification. After hydrolysis Kylin isolated phenyl-L-fucosazone and claimed that pentoses were present also in the hydrolysate.

Bird and Haas (*Biochem. J.*, 1931, **25**, 403) obtained fucoidin by soaking fresh fronds of *Laminaria digitata* in water and precipitating a crude sulphate from the viscous extract with ethanol. After suitable treatment a product containing 30·9% of ash (chiefly calcium sulphate) and 30·3% of sulphate was obtained. Since the total sulphate content was approximately double that found in the ash, fucoidin was designated a carbohydrate ethereal sulphate. Bird and Haas (*loc. cit.*) reported the presence of uronic acid (7·3%) in their preparation.

Lunde, Heen, and Öy (*Z. physiol. Chem.*, 1937, **247**, 189) allowed the droplets exuding from freshly-gathered *Laminaria digitata* to fall into ethanol, and, after its purification from boiling ethanol, obtained a specimen with an ash content of 26—30% of which the sulphate content was approximately half the total estimated in the polysaccharide. In this specimen the principal inorganic ion was sodium. Estimations by distillation with hydrochloric acid gave 33—37% of methylpentose, and since this failed to account for more than 80% of the carbo-

hydrate constituents a formula $(R{\cdot}R'{\cdot}O{\cdot}SO_2{\cdot}OM)_n$ was proposed where R is fucose (as $C_6H_{10}O_4$), R′ is unknown, and M may be Na, K, $Ca_{0\cdot5}$ or $Mg_{0\cdot5}$.

Another water-soluble polysaccharide closely related to, if not identical with, fucoidin was first isolated from the giant kelp *Macrocystis pyrifera* (Hoagland and Lieb, *J. Biol. Chem.*, 1915, **23**, 287) and shown to contain L-fucose and a high proportion of calcium and sulphate. Further studies by Nelson and Cretcher (*ibid.*, 1931, **94**, 147) revealed that the sulphate existed in the form of ester groupings, and that the only sugar identified after hydrolysis was L-fucose, although contamination with alginates was suspected on the ground of a uronic acid content of 2·6%.

In an attempt to reconcile some of the conflicting views on the nature of fucoidin, specimens have been prepared from *Fucus vesiculosus*, *Fucus spiralis*, *Laminaria cloustoni*, and *Himanthalia lorea*. The method adopted was by extraction with hot water, removal of alginates and proteins with lead acetate, and addition of barium hydroxide. The resulting lead hydroxide–fucoidin complex was then decomposed with dilute sulphuric acid, and the polysaccharide isolated after prolonged dialysis, although several treatments with "Filter Cel" and precipitation from ethanol were necessary before anything approaching a pure product was obtained. The presence of a sulphate ester was confirmed in the usual way for all our

Source.	Fucose, %.	SO_4, %.	Metals, %.	Ca, %.	$[\alpha]_D^{15}$.
F. vesiculosus	37·4	31·7	—	—	−119°
F. spiralis	35·8	32·7	6·4	5·8	−118
L. cloustoni	36·5	33·5	7·1	6·1	−114
F. vesiculosus	41·2	33·6	6·5	—	—
Himanthalia lorea	43·9	32·4	6·9	—	−140

specimens; in contrast to the findings of Lunde *et al.* (*loc. cit.*) the main inorganic ion was found to be calcium, a typical ash analysis giving Ca 25·0, Mg 1·9, Na 1·1, K 0·8, and SO_4 73·2%. The earlier specimens examined contained glucose, presumably owing to the incomplete removal of laminarin. In the Table the results for a number of specimens of fucoidin are set out. Fucose was estimated by the method of Cameron, Ross, and Percival (*J. Soc. Chem. Ind.*, 1948, **67**, 161) although it was found later that slightly higher values were obtained by chromatographic analysis.

Since a calcium fucosan sulphate would give 66·9% of fucose on hydrolysis it appeared that the view held by Lunde had some justification. Nevertheless a prolonged search by various methods, including periodate oxidation of hydrolysis products and chromatographic analysis, failed to reveal the presence of more than small quantities of other sugars. The discrepancy was accounted for when it was realised that despite drying at 40°/0·1 mm. for 18 hours, the polysaccharide stubbornly retained water (9·4%) and ethanol (6%) as revealed by micro-Zerewitinow determinations and by the use of the method of Newman (*J. Pharmacol.*, 1936, **56**, 278) for the determination of ethanol.

This result recalls the observations of Berner (*Ber.*, 1931, **64**, 842) on the difficulty of removing adsorbed ethanol from inulin, and those of Jansen, MacDonnell, and Ward (*Arch. Biochem.*, 1949, **21**, 149) on the retention of ethanol by polygalacturonic esters. In the present instance the attraction for water is probably enhanced by the presence of the sulphate residues.

After making due allowances for the adsorbed solvents, and by chromatographic estimations of the reducing sugars present, the following result was obtained for the most highly purified specimen of *Himanthalea lorea* fucoidin: SO_4, 38·3; metals, 8·2; uronic acid, 3·3; fucose on hydrolysis, 56·7; galactose, 4·1; xylose, 1·5%. A calcium fucosan monosulphate $(C_6H_9O_3,SO_4Ca_{0\cdot5})_n$ would give: SO_4, 39·2; Ca, 8·2; fucose 66·9%.

The simplest interpretation of these results is that the principal constituent of the fucoidin examined is a fucosan monosulphate and that the other constituents arise from adventitious impurities.

Experimental.

Isolation of Crude Fucoidin from Seaweeds.—Samples of crude fucoidin were prepared from *Fucus vesiculosus*, *F. spiralis*, *Laminaria cloustoni*, and *Himanthalea lorea* as follows: For the first specimens, dried ground seaweed (1 kg.) was extracted with hot water (3 l.) for 24 hours at the temperature of a boiling water-bath and the residue removed by filtration through muslin. The extract was treated with lead acetate solution until precipitation of alginates and proteins was complete. After filtration the solution was made just alkaline to phenolphthalein with barium hydroxide, yielding a lead hydroxide–fucoidin complex which was removed and decomposed with dilute sulphuric acid (300 c.c. of 4N-acid in 1 l. of water) by shaking overnight, followed by dialysis for a week in a Cellophane bag to remove the acid, filtration, evaporation (40°/15 mm.) to small bulk, and precipitation in alcohol. The resulting solid, which was washed with alcohol and ether and dried over phosphoric oxide in a vacuum,

was buff-coloured and a representative analysis was: fucose (by the method of Cameron, Ross, and Percival, *loc. cit.*), 29·6; SO_4, 27·3%. Yields: *F. vesiculosus*, 23 g.; *F. spiralis*, 18 g.; *L. cloustoni*, 8 g.

Since the determination of fucose required the preliminary hydrolysis of the polysaccharide, the rate of hydrolysis of the sample from *F. vesiculosus* was followed polarimetrically; hydrolysis was complete in 3 hours in 0·5N-sulphuric acid, the rotation rising from $[\alpha]_D^{15}$ −119° to −24°.

Further Purification of Fucoidin.—Dissolution in water, followed by evaporation and reprecipitation in alcohol, did not increase the fucose content but, if the solution in water was set aside overnight with 2% Johns-Manville "Filter Cel" and then filtered and the product was isolated as before, the fucose released on hydrolysis of the sample noted before rose to 35·4% and a second treatment increased this figure to 37·4%, the total sulphate rising to 31·7%. The samples from other species of seaweed gave similar figures when treated in the same way although these operations involved serious loss of material. Sulphate, metals, and $[\alpha]_D$ were also determined (see foregoing Table).

The sample of crude polysaccharide prepared from *Himanthalea lorea* was extracted from freshly gathered, minced seaweed (50 kg.) as before except that, after decomposition of the lead hydroxide precipitate with acid, the mixture was filtered through "Filter Cel" which removed much colouring matter. The fucoidin (190 g.) was separated after concentration and precipitation in alcohol. This product (20 g.) was redissolved in water and treated twice with charcoal (1%; Merck) and "Filter Cel" (2%) at 90° for ½ hour, yielding after filtration and isolation a pure white product (12 g.) containing fucose 43·9%, SO_4 32·4%, ash 22·6%, and metals 6·9%. This sample, which was used for the later analyses unless otherwise stated, was the most highly purified specimen obtainable by this method, two additional treatments giving a product with the same analytical figures and $[\alpha]_D^{5}$ −140° (*c*, 1·0 in water).

Investigation of Other Components of Crude Fucoidin.—Before the fucoidin was obtained from *H. lorea*, some investigations were made into the undetermined portion of the polysaccharide from *F. vesiculosus*.

Estimation of pentose (Marshall and Norris, *Biochem. J.*, 1937, **31**, 1289, 1939) and uronic acid (MacCready, Swenson, and Maclay, *Ind. Eng. Chem. Anal.*, 1946, **18**, 290) gave figures of 3·4% and 6·6% respectively, but no free sugars or methoxyl groups could be detected. Hypoiodite oxidation after hydrolysis, on the other hand, indicated about 20% of sugars as hexose after allowance for fucose, by addition of excess of alkaline hypoiodite and titration of the excess with thiosulphate after storage for 1 hour and acidification. Oxidation experiments using periodic acid were also carried out after preliminary investigations into the oxidation of glucose and rhamnose. On treatment with periodic acid pentoses and hexoses yield 1 molecule of formaldehyde along with formic acid (Reeves, *J. Amer. Chem. Soc.*, 1941, **63**, 1476), the formaldehyde being estimated as the dimedon complex (Vorländer, *Z. Analyt. Chem.*, 1929, **77**, 241). In the present case glucose was found to give 0·96 mol. of formaldehyde when treated in admixture with rhamnose, provided that the acetaldehyde formed from the methylpentose was removed by aeration before precipitation with dimedon. The formic acid liberated was also determined by titration after destruction of the excess of periodate with ethylene glycol, glucose giving 4·90 mols. and rhamnose 3·99 mols. of formic acid. When applied to fucoidin, the production of formaldehyde was equivalent to 19·1% of hexose, and that of formic acid after allowance for fucose to 14·8% of hexose.

A sample of fucoidin was hydrolysed and the fucose mainly removed as the phenylmethylhydrazone, the remaining material, after treatment with benzaldehyde (Lüdtke, *Biochem. Z.*, 1929, **212**, 419), being finally obtained as a glass. A small amount of alcohol-insoluble material was shown to be mainly uronic acid whilst the soluble portion contained fucose 5·4% and hexose (by periodate oxidation) 51·9%. This gave glucosazone (m. p. 186°) which yielded phenyl-D-glucosotriazole (Haskins, Hann, and Hudson, *J. Amer. Chem. Soc.*, 1945, **67**, 939) (m. p. 196—197°, mixed m. p. 193—194°, $[\alpha]_D^{15}$ −80° in pyridine). These results indicate the presence of glucose since it was found impossible to obtain mannose phenylhydrazone under conditions in which a control mannose solution reacted readily. Estimation of the glucose by the Somogyi micro-copper method (*J. Biol. Chem.*, 1945, **160**, 61) before and after incubation with yeast at 37° (Harding and Selby, *Biochem. J.*, 1931, **25**, 1815) indicated the presence of 15·8% of glucose in the glass along with a similar amount of another reducing sugar, not absorbed by yeast, which was not identified.

Properties of Fucoidin as a Free Acid.—Freshly prepared fucoidin solution (0·2%) had a pH value of 6·1 which fell to 4·0 on storage for 1 week owing to autohydrolysis. Fucoidin (0·35 g.) was dissolved in water (150 c.c.), and the solution titrated electrometrically with sodium hydroxide (0·01N.) with a platinum–quinhydrone–N-calomel cell. After titration to a pH of 7·26, the calculated amount of oxalic acid necessary to precipitate exactly the calcium present was added and the titration of the free acid continued using sodium hydroxide (0·1N.). The results in the following table indicate the presence of a strong acid.

C.c. of NaOH (0·1045N.) added ...	—	1·0	3·0	5·0	6·0	7·0	9·0	9·5	9·6	9·7
pH ...	3·72	3·74	3·91	4·45	4·63	4·96	5·79	6·84	7·16	7·26

Further Analyses of the Purest Fucoidin from H. lorea.—All analyses were carried out on samples dried at 40°/0·1 mm. for 16 hours. This sample contained fucose 43·9% and SO_4 32·4%. Quantitative paper chromatographic analysis (Flood, Hirst, and Jones, *J.*, 1948, 1679) on this material, after hydrolysis, gave fucose 48·4%, which is slightly higher than that obtained by the volumetric estimation. Fucoidin (1·247 g.) was hydrolysed with sulphuric acid (100 c.c.; N.) and neutralised, and the free sugars were obtained as a glass, all the operations being carried out as far as possible quantitatively. The glassy product (0·755 g. or 60·5%) had $[\alpha]_D^{15}$ −54·8 (*c*, 1·26 in water), fucose 75·0%, sulphate as calcium sulphate 7·8%, and sulphated ash 7·2%. A quantitative paper chromatogram on the glass gave fucose 79·1% and galactose 7·2%, representing 48·0% and 4·4% respectively calculated on the polysaccharide. Development of a chromatogram with aniline oxalate solution confirmed the presence of hexose (brown spot) and also indicated a small quantity of a pentose (pink spot) corresponding to

xylose. A second quantitative determination using more material was carried out and gave galactose amounting to 5·8% and xylose 2·2% of the glass.

The analytical figures to date on the polysaccharide were fucose 48·0%, galactose 3·5%, xylose 1·3%, uronic acid 2·8%, sulphate 32·4%, and metals 6·9%. Calculation of these figures to anhydro-sugars, etc., as in the polysaccharide showed that about 15% remained to be accounted for.

Alcohol determinations were carried out by the method of Newman (*J. Pharmacol.*, 1936, **56**, 278) on samples dried for 18 hours at 40°/0·1 mm. The alcohol is removed from the sample by evacuation after dissolution in water and addition of anhydrous sodium sulphate, and is absorbed in acid dichromate solution; the excess of dichromate is then titrated with sodium thiosulphate in the presence of potassium iodide. Found : adsorbed alcohol 6·0% (average of three determinations). Adsorbed water was also investigated and determined by means of the Zerewitinow reaction (*Ber.*, 1907, **40**, 2033) modified to the micro-determination of active hydrogen (Flaschenträger, *Z. physiol. Chem.*, 1925, **146**, 219; Pregl–Grant, " Quantitative Organic Micro-analysis," 4th Edn., 1945), allowance being made for the active hydrogen in the ethanol in the polysaccharide. It was found that methane was produced equivalent to 9·5% of water in the polysaccharide. The possibility of other reactions taking place was negatived by a similar determination on starch with a known moisture content of 12·1% (Found : water 12·7%). By weighing a supposedly dry sample of fucoidin at intervals after storage in an atmosphere of 60% relative humidity, it was found that after 1 day the moisture content increased by 17% and, after 6 days, by 26%, showing that intensive drying before analysis is necessary and confirming the possibility of fucoidin's high power of retaining water after being dried by the normal methods.

The addition of water and alcohol to the previous analytical figures brings the total percentage accounted for to 99·3%.

Thanks are expressed to the Scottish Seaweed Research Association for permission to publish this work.

KING'S BUILDINGS, UNIVERSITY OF EDINBURGH. [*Received, December 13th*, 1949.]