141. *The Structure of Laminarin. Part I. The Main Polymeric Linkage.*

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The linkages in insoluble and soluble laminarin have been analysed by the method of partial hydrolysis. It is confirmed that the principal linkage is of the 1 : 3-type, between β-D-glucopyranose residues. The presence of other (minor) structural features in both forms of the polysaccharide is indicated. Some properties of the new sugars, laminari-triose, -tetraose, and -pentaose are reported.

The polysaccharide laminarin is a reserve carbohydrate of the sublittoral brown algae (Phaeophyceae) and occurs principally in the *Laminariae*, the species from which most investigators have obtained their material.[1-5] Two types of laminarin are recognised; the water-insoluble laminarin, which is precipitated spontaneously from an aqueous acid extract of the weed,[6] and the soluble form, which separates from solution only on the addition of a precipitant, such as ethanol.[7,8] Both forms are reported to consist entirely of D-glucose residues [8-10] and no clear-cut differences in structure have been established.[8,9]

The character of the main, if not the only, polymeric linkage in insoluble laminarin was established first by Barry,[10] who isolated 2 : 4 : 6-tri-*O*-methyl-D-glucose in large yield from the hydrolytic products of the methylated polysaccharide. The chain-forming 1 : 3-linkage probably has the β-configuration because of the negative optical rotation of the polysaccharide and its derivatives.[8-11] Later workers [8,9] isolated the same trimethyl sugar together with smaller amounts of 2 : 3 : 4 : 6-tetra-, 2 : 6-di-, and 4 : 6-di-*O*-methyl-glucoses from both forms of methylated laminarin. Judgment on the origin of the dimethyl sugars was reserved since experiments showed that these same substances could arise by acid treatment of the trimethyl sugar. Accordingly, both forms of laminarin were depicted as linear chains of β-1 : 3-linked glucose residues, the proportion of tetramethylglucose obtained suggesting a chain length of about 20 glucose units in each case. Determination of the molecular weight by other methods gave results which were discordant among themselves and which did not agree with the estimate made by

[1] Black, *J. Soc. Chem. Ind.*, 1948, **67**, 165, 169, 172.
[2] Black, *Nature*, 1948, **161**, 174.
[3] Black, *J. Mar. Biol. Assoc. U.K.*, 1950, **29**, 45.
[4] Black, *J. Soc. Chem. Ind.*, 1950, **69**, 161.
[5] Black and Dewar, *J. Mar. Biol. Assoc. U.K.*, 1949, **28**, 673, 687.
[6] Barry, *Sci. Proc. Roy. Dublin Soc.*, 1938, **21**, 615.
[7] Black, Cornhill, Dewar, and Woodward, *J. Appl. Chem.*, 1951, **1**, 505.
[8] Percival and Ross, *J.*, 1951, 720.
[9] Connell, Hirst, and Percival, *J.*, 1950, 3494.
[10] Barry, *Sci. Proc. Roy. Dublin Soc.*, 1939, **22**, 59.
[11] Bächli and Percival, *J.*, 1952, 1243.

methylation assay.[8,9] Confirmatory evidence of the polysaccharide structure was provided by the isolation of a disaccharide (laminaribiose) from enzymic and acidic hydrolysates of insoluble laminarin.[11,12] Chemical synthesis[11,13] showed this to be 3-*O*-β-D-glucopyranosyl-D-glucose. The resistance of laminarin to periodate oxidation[14,15] is also consistent with 1 : 3-bonding and the supposed β-configuration of the linkage was confirmed by the hydrolytic action of specific β-glucosidases on laminarin.[16–18]

Various hypotheses have been advanced to explain the different physical properties of the two forms of laminarin[6,10,19,20] but detailed structural investigation has revealed no chemical difference except in regard to its reducing action rowards alkaline hypoiodite.[8,9] Soluble laminarin has a higher weight-average molecular weight than the insoluble form but this is not believed to be the sole cause of the different solubilities.[21] As a result of physical and chemical investigations Broatch and Greenwood[22] have recently concluded that laminarin (of unspecified source) has a branched molecule. This is in line with our preliminary report[23] that β-1 : 6-links, which may represent points of branching, are present in both forms of laminarin.

Our interest in laminarin arose from the need to obtain the series of β-1 : 3-linked glucose oligosaccharides (laminaridextrins) as reference compounds. These substances would be the components of a partial acid hydrolysate of laminarin, if it is constituted as described above, and should be capable of isolation by charcoal–chromatographic fractionation. When this procedure was carried out on a sample of insoluble laminarin it at once became obvious that although glucose and the laminaridextrins were the main components of the mixture, other products were present in amounts which suggested that they represented significant and previously unrecognised features of the laminarin molecule. Accordingly, detailed investigations of both forms of the polysaccharide were undertaken by the method of linkage analysis.[24] For convenience of presentation, this paper describes the characterisation of the main hydrolysis components. Part II (following paper) deals in detail with the minor components.

Insoluble Laminarin.—The polysaccharide (22 g.) was partly hydrolysed in hot 0·33N-sulphuric acid and the products fractionated on charcoal–Celite. The monosaccharide fraction consisted mainly of glucose (3·62 g.), characterised as the β-penta-acetate, together with mannitol.[25] The higher-molecular weight groups were similar mixtures of major and minor conponents. In general the minor components were eluted before the major component of the same molecular weight.

The major di- to penta-saccharide components were obtained in chromatographically pure form and their specific optical rotations and R_M values were determined. Crystalline β-acetates were also prepared from the di-, tri-, and tetra-saccharides (see Table 1).

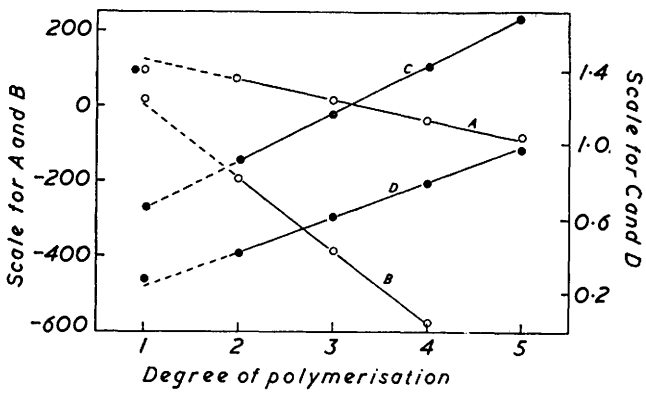
The disaccharide and its acetate had properties identical with those previously reported for laminaribiose.[11,13] When the R_M values of the sugars or the molecular rotations of the sugars and their acetates were plotted against degree of polymerisation (known for the disaccharide and assumed for the higher oligosaccharides) linear relations were observed in all cases (see Figure). This behaviour characterises the tri- and higher saccharides as component members of the series of laminaridextrins. Further proof of the identity of

[12] Barry, *Sci. Proc. Roy. Dublin Soc.*, 1941, **24**, 423.
[13] Freudenberg and Oertzen, *Annalen*, 1951, **574**, 37.
[14] Barry, Dillon, and McGattrick, *J.*, 1942, 183.
[15] Barry, *J.*, 1942, 578.
[16] Peat, Thomas, and Whelan, *J.*, 1952, 722.
[17] Dillon and O'Colla, *Nature*, 1950, **166**, 67.
[18] Dillon and O'Colla, *Chem. and Ind.*, 1951, 111.
[19] Kylin, *Z. physiol. Chem.*, 1913, **83**, 171; 1915, **94**, 337.
[20] Gruzewska, *Bull. Soc. Chim. biol.*, 1923, **5**, 216.
[21] Friedlaender, Cook, and Martin, *Biochim. Biophys. Acta*, 1954, **14**, 136.
[22] Broatch and Greenwood, *Chem. and Ind.*, 1956, 1015.
[23] Peat, Whelan, and Lawley, *Biochem. J.*, 1953, **54**, xxxiii.
[24] Peat, Whelan, and Edwards, *J.*, 1955, 355.
[25] Peat, Whelan, Lawley, and Evans, *Biochem. J.*, 1955, **61**, x.

these substances came from paper-chromatographic examination of their partial acid hydrolysates. The trisaccharide gave rise to glucose and a single disaccharide (laminaribiose), the tetrasaccharide to a single di- and tri-saccharide and the pentasaccharide to a single tetrasaccharide, etc. In addition, laminaribiose was a product of the partial degradation of the trisaccharide by almond emulsin (β-glucosidase).

Soluble Laminarin.—A large-scale partial acid hydrolysate (27 g.) was prepared and fractionated as for insoluble laminarin. Only the mono-, di-, and tri-saccharide fractions were examined. It was again observed that each fraction contained more than one component and the amounts of the minor components were greater in proportion to the major components than for insoluble laminarin (see Part II). The properties of the major products (glucose, laminaribiose, and laminaritriose) are listed in Table 2. Products which had not been isolated from insoluble laminarin were L-fucose (0·39 g.), characterised as the phenylhydrazone, and traces of xylose and galactose. It is very probable that these arose by contamination of the soluble laminarin with fucoidin. A similar observation

Physical properties of the laminaridextrins and their β-acetates. A, Molecular rotations (°) of free sugars; B, molecular rotations (°) of β-acetates; C, D, R_M values of free sugars in solvents 1 and 2, respectively (see Experimental section).



was previously made by Percival and Ross.[26] No oligosaccharides containing any of these three sugars were detected and it is not considered that they form part of the soluble laminarin molecule.

Conclusions.—These results prove that insoluble and soluble laminarin consist mainly of β-D-glucose residues joined continuously through 1 : 3-linkages and are entirely consistent with previous methylation studies.[8-10] The application of the technique of linkage analysis to the problem has, however, revealed previously unsuspected features of the structures of the two forms of the polysaccharide which merited further examination as described in the following paper.

Experimental

Analytical Methods.—Paper chromatography was carried out on Whatman No. 1 or No. 54 paper; the irrigating solvents were (1) butan-1-ol–acetic acid–water (4 : 1 : 5, by vol.) or (2) butan-1-ol–pyridine–water (6 : 4 : 3, by vol.). Solutions of the sugars prepared from laminarin were treated before analysis with Somogyi's deproteinising reagents [27] (cf. ref. 28). Values of specific optical rotation of laminarin and the derived oligosaccharides are based on concentrations calculated from the glucose released on complete hydrolysis with acid,[29] together with rotations measured in a 4-dm. tube. All evaporations were made under diminished pressure at 40°, the aqueous solutions being kept at pH 5 by addition of sodium hydroxide or acetic acid.

[26] Percival and Ross, *J.*, 1950, 717.
[27] Somogyi, *J. Biol. Chem.*, 1945, **160**, 69.
[28] Whelan, Bailey, and Roberts, *J.*, 1953, 1293.
[29] Pirt and Whelan, *J. Sci. Food Agric.*, 1951, **2**, 224.

Insoluble Laminarin

Properties of the Polysaccharide.—Insoluble laminarin was isolated from *L. cloustoni* as by Black *et al.*[7] and was kindly provided by Dr. E. T. Dewar. The dry material contained 92·5% of polyglucose [29] and 0·4% of non-volatile matter, and had $[\alpha]_D^{16}$ −13·4° (*c* 0·9 in water). The amorphous triacetate (52% yield; method of Percival and Ross [8]) had $[\alpha]_D^{16}$ −63·5° (*c* 0·4 in chloroform) [Found: Ac, 43·2. Calc. for $(C_{12}H_{16}O_8)_n$: Ac, 44·8%]. After complete hydrolysis (1·5N-sulphuric acid; 2 hr.; 100°) and neutralisation (barium carbonate), examination with benzidine spray [30] of the paper chromatograms (solvents 1 and 2) revealed glucose as the only reducing component, but silver nitrate–sodium hydroxide,[31] which detects reducing and non-reducing carbohydrates, revealed glucose and a substance of slightly greater R_F value.

Partial Acid Hydrolysis.—Isolation of the laminaridextrins. In a preliminary experiment, laminarin (264 mg.) was hydrolysed in 0·33N-sulphuric acid (25 ml.) at 100° and portions were removed at intervals for determination of copper-reducing power.[32] After 2 hr. the apparent conversion into glucose was 39·0%, at which level a good yield of low-molecular-weight oligosaccharides was expected (cf. ref. 28). Accordingly, the large-scale hydrolyses were carried to this degree. Laminarin (11·04 g.) was dissolved in warm water (500 ml.) and diluted (to 1 l.) with 5N-sulphuric acid (66 ml.) and water. After 2 hr. at 100° the solution was poured on ice, brought to pH 8 with sodium hydroxide, and then made slightly acid (pH 5). The same procedure was repeated exactly and the hydrolysates combined. The apparent conversion into glucose was 33·8%. The solution was evaporated to dryness and a portion of the residue (50 mg.) was freed from electrolyte by extraction with pyridine.[33] Examination of the sugars by paper chromatography (solvent 1) revealed glucose and a series of reducing oligosaccharides. Less prominent were the non-reducing substance previously detected in the fully hydrolysed laminarin, and a series of non-reducing oligosaccharides. The remainder of the residue was dissolved in water (100 ml.) and much of the sodium sulphate removed by filtration. The filtrate and washings were adsorbed on charcoal–Celite (115 × 4 cm.) and the sugars eluted with water and stepwise-increasing concentrations of ethanol in water.[28] After suitable combination, the fractions were evaporated to dryness, the sugars dissolved in 80% methanol to eliminate inorganic matter, and the solutions re-evaporated. Paper-chromatographic fractionation (solvent 1) showed that the principal tri- and tetra-saccharide fractions were single substances. The main di- and penta-saccharide fractions were mixtures, however, and were refractionated by charcoal–Celite and paper (Whatman No. 3) chromatography, respectively. The monosaccharide fraction was mainly glucose, with a trace of the faster-moving non-reducing component. The yields of major products are shown in Table 1. In addition a non-reducing disaccharide (256 mg.), a non-reducing trisaccharide (*ca.* 100 mg.), gentiobiose (82 mg.), isomaltose (*ca.* 25 mg.), and several reducing trisaccharides other than laminaritriose (*ca.* 20 mg. of each) were isolated. Discussion of the significance of these minor components is reserved for the following paper.

Acid and Enzymic Hydrolysis of the Major Tri-, Tetra-, and Penta-saccharides.—A portion (*ca.* 20 mg.) of each sugar was hydrolysed in 0·33N-sulphuric acid (2 ml.) at 100° for 30 min., the solution neutralised with barium carbonate, and the supernatant liquid examined by paper chromatography in solvent 1. The trisaccharide yielded only laminaribiose and glucose; the tetrasaccharide gave laminaribiose and glucose and the trisaccharide, while the pentasaccharide gave rise to the tetra- and tri-saccharides, with laminaribiose and glucose. It seemed clear, therefore, that the tri-, tetra-, and penta-saccharides were laminari-triose, -tetraose, and -pentaose, respectively.

The trisaccharide (10 mg.) was incubated at 35° with a suspension of almond emulsion [34] (10 mg.) in water (1 ml.) for 1·5 hr. After the digest had been heated at 100° the supernatant solution was examined by paper chromatography (solvent 1). The substances located had R_F values corresponding to the trisaccharide, laminaribiose, and glucose.

Acetates of Glucose and the Laminaridextrins.—Acetylation of the sugar (50—200 mg. quantities) was carried out with sodium acetate–acetic anhydride and the products were worked up in the usual way, being finally dissolved in ethanol, decolorised with charcoal, and allowed

[30] Bacon and Edelman, *Biochem. J.*, 1951, **48**, 114.
[31] Trevelyan, Proctor, and Harrison, *Nature*, 1950, **166**, 444.
[32] Somogyi, *J. Biol. Chem.*, 1945, **160**, 61.
[33] Malpress and Morrison, *Nature*, 1949, **164**, 963.
[34] Tauber, *J. Biol. Chem.*, 1932—33, **99**, 257.

to crystallise. The yields varied from 74 to 90%. The pentasaccharide acetate failed to crystallise. The properties of the derivatives are listed in Table 1. Elementary analyses gave the following results. β-Laminaribiose octa-*O*-acetate (Found: C, 49·5; H, 5·7. Calc. for $C_{28}H_{38}O_{19}$: C, 49·6; H, 5·6%), *β-laminaritriose hendeca*-O-*acetate* (Found: C, 48·9; H, 5·8.

TABLE 1. *Properties of the main components of partly hydrolysed insoluble laminarin.*

	Sugar				β-Acetate	
			R_F value [2]			
Substance	Yield [1] (g.)	$[\alpha]_D^{17-21}$ in H_2O	Solv. 1	Solv. 2	M. p.	$[\alpha]_D^{17-21}$ in $CHCl_3$
Glucose	3·62	52·7°	0·18	0·35	132—133°	3·8°
Laminaribiose	2·67	20·4	0·11	0·28	160—161	−28·6
Laminaritriose	2·02	2·4	0·065	0·20	120—121	−40·0
Laminaritetraose	1·6	− 5·9	0·038	0·14	122—123	−46·2
Laminaripentaose	1·3	−10·3	0·021	0·097	—	—

[1] From 22·08 g. of polysaccharide. The weights are those obtained in the first fractionation.
[2] Solvents are defined in the Experimental section.

$C_{40}H_{54}O_{27},H_2O$ requires C, 48·8; H, 5·7%), and *β-laminaritetraose tetradeca*-O-*acetate* (Found: C, 48·45; H, 5·8%. $C_{52}H_{70}O_{35},H_2O$ requires C, 49·1; H, 5·7%; $C_{52}H_{70}O_{35},2H_2O$ requires C, 48·4; H, 5·7%).

Soluble Laminarin

Soluble laminarin was isolated from *L. digitata* as by Black *et al.*[7] and was a gift from Dr. E. T. Dewar. The dried polysaccharide contained 91·2% of polyglucose [29] and 1·0% of non-volatile matter, and had $[\alpha]_D^{18}$ −11·9° (*c* 2·1 in water). The tri-*O*-acetyl derivative [8] had $[\alpha]_D^{17}$ −64·6° (*c* 0·9 in chloroform) [Found: Ac, 43·9. Calc. for $(C_{12}H_{16}O_8)_n$: Ac, 44·8%]. After complete acid hydrolysis, as for insoluble laminarin, paper chromatography revealed glucose with much smaller amounts of the same non-reducing component and fucose. The products of partial acid hydrolysis were the same as for insoluble laminarin, with fucose as an additional product.

Large-scale Acid Hydrolysis.—The rate of acid hydrolysis was the same as for insoluble laminarin. Accordingly, the polysaccharide (26·95 g.) was hydrolysed in 0·33N-sulphuric acid for 2 hr. at 100° and the hydrolysate (38·4% conversion) worked up and fractionated as previously described for insoluble laminarin, except that elution from the charcoal was discontinued after the emergence of the principal trisaccharide component.

The monosaccharide fraction contained glucose (see Table 2) with a much smaller amount of the non-reducing component, traces of xylose and galactose (identified by paper chromatography), and L-fucose (0·39 g.). The derived fucose phenylhydrazone had m. p. 166—167° (Found: C, 56·7; H, 7·5; N, 10·7. Calc. for $C_{12}H_{18}O_4N_2$: C, 56·7; H, 7·1; N, 11·0%). It was necessary to refractionate the disaccharide fraction on charcoal–Celite in order to obtain pure specimens of laminaribiose (Table 2), the non-reducing disaccharide, and gentiobiose. Isomaltose was not detected. As with insoluble laminarin the trisaccharide fraction consisted of a major component (laminaritriose) and minor components, *viz.*, a non-reducing trisaccharide and reducing trisaccharides. All these minor components are discussed in detail in the following paper.

TABLE 2. *Properties of the main components of partly hydrolysed soluble laminarin.*

	Sugar [1]		β-Acetate	
	Yield [2] (g.)	$[\alpha]_D^{14-19}$ in H_2O	M. p.	$[\alpha]_D^{19-20}$ in $CHCl_3$
Glucose	5·82	50·9°	132—133°	3·8°
Laminaribiose	2·22	18·6	162—163	−28·9
Laminaritriose	2·41	2·0	120—121	−40·1

[1] R_F values were the same as in Table 1. [2] From 26·95 g. of polysaccharide. The weights of glucose and laminaritriose are those from the first fractionation, that of laminaribiose is after refractionation.

The glucose, and laminari-biose and -triose were identified by measurement of the physical properties of the sugars and their β-*O*-acetyl derivatives (Table 2).

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