142. *The Structure of Laminarin. Part II.*[1] *The Minor Structural Features.*

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Insoluble laminarin has been partly hydrolysed by acid, and the products fractionated. Fourteen mono-, di-, and tri-saccharide products have been identified and of these, D-glucose, D-mannitol, laminaribiose, gentiobiose, 1-*O*-β-glucosylmannitol, laminaritriose, 3-*O*-gentiobiosylglucose, 6-*O*-β-laminaribiosylglucose, and 1-*O*-β-laminaribiosylmannitol are considered to be true structural fragments of laminarin. It is suggested that the laminarin molecules may consist of unbranched chains of β-1 : 3-linked glucose units, occasionally interrupted by β-1 : 6-links, and that some, but not all, of the chains are terminated by mannitol. Soluble laminarin probably has a similar structure but contains a higher proportion of mannitol and of β-1 : 6-links. A method for the quantitative determination of mannitol in laminarin is described.

PREVIOUS work on the structure of laminarin has been summarised in the preceding paper,[1] in which confirmatory evidence was provided that the main polymeric linkage in this β-glucan is of the 1 : 3-type. Our investigations did not, however, confirm the view that laminarin is constituted solely of glucose residues or that the β-1 : 3-linkage is the only type present. We now report on additional structural features encountered in the two forms of laminarin, insoluble and soluble. Most of the work has been concerned with the insoluble form.

The conception of laminarin as a 1 : 3-linked β-glucan implies that a partial acid hydrolysate would contain one monosaccharide (glucose), one disaccharide (laminaribiose), and one trisaccharide (laminaritriose). We have actually isolated and characterised two monosaccharide components, three disaccharides, and four trisaccharides, in yields which seem to preclude the possibility that they are acid-reversion products. Laminarin cannot therefore be the simple polymer envisaged by earlier investigators.

The first indication of the complexity of the laminarin molecule came from an examination of the disaccharide fraction of the partial acid hydrolysate of insoluble laminarin (22·08 g.) in which were identified gentiobiose (82 mg.), isomaltose (25 mg.) and a non-reducing disaccharide (256 mg.) in addition to the laminaribiose (2·67 g.) previously reported.[1] The non-reducing substance was at first incorrectly identified as ββ-trehalose,[2] owing to a fortuitous coincidence of certain properties, in particular, specific optical rotation and R_F value. The product was subsequently proved to be 1-*O*-β-D-glucopyranosyl-D-mannitol. A systematic study was therefore made of the components of an acid hydrolysate of a larger sample (137 g.) of insoluble laminarin.

Mannitol and the Non-reducing Oligosaccharides.—A search for mannitol in the monosaccharide fraction of the laminarin hydrolysate was first undertaken. The separation of glucose and mannitol by chemical means proved impracticable owing to the great excess of glucose and resort was had to the removal of the glucose by yeast fermentation. D-Mannitol (as the hexa-acetate) was obtained from the non-fermentable residue (see Table).

Crystalline mannitol hexabenzoate was later isolated from the acid hydrolysate of the non-reducing disaccharide mentioned above. The identity of this disaccharide with the 1-*O*-β-glucosylmannitol, previously found by Lindberg[3] in uncombined form in *Fucus vesiculosus*, was established as follows: (1) β-Glucosidase released glucose and mannitol.

[1] Part I, preceding paper.
[2] Peat, Whelan, and Lawley, *Biochem. J.*, 1953, **54**, xxxiii.
[3] Lindberg, *Acta Chem. Scand.*, 1953, **7**, 1119.

(2) The sugar (1 ml.) consumed 5·9 mol. of periodate and yielded 3·8 mol. of formic acid, the calculated values being 6 and 4 mol., respectively. (3) The disaccharide and an authentic sample of 1-*O*-β-glucosylmannitol had the same specific optical rotation, R_F value, and M_G value, and formed identical crystalline nona-*O*-benzoates (see Table).

In examining the "trisaccharide" fraction for non-reducing compounds, attention was concentrated on the four "trisaccharides" which could theoretically give rise to the "disaccharides" found in the hydrolysate, namely, laminaribiose, gentiobiose, isomaltose, and the glucosylmannitol. These non-reducing "trisaccharides" would include 1 : 6-di-*O*-β-glucosylmannitol. This would yield, on partial hydrolysis, 1- and 6-*O*-β-glucosylmannitols which, because of the central symmetry of mannitol, are alternative names for the same compound. Three of the four possible non-reducing substances were isolated from the "trisaccharide" fraction, namely, 1-*O*-β-laminaribiosylmannitol (1·1 g.), 1 : 6-di-*O*-β-glucosylmannitol (0·04 g.), and 1-*O*-β-isomaltosylmannitol (0·01 g.). The last two named components were not further examined because the yields in which they were obtained indicated that they might be artefacts produced by acid reversion. The laminaribiosylmannitol, however, was of obvious structural significance. It was identified on the following evidence: (1) Laminaribiose and 1-*O*-β-glucosylmannitol were the "disaccharide" products of its partial acid hydrolysis. (2) β-Glucosidase split off glucose to yield glycosylmannitol. (3) Mannitol isolated from a complete acid hydrolysate corresponded to 0·98 mole per mole of "trisaccharide." (4) The uptake of periodate (6·08 mol.) and the concomitant release of formic acid (3·72 mol.) and formaldehyde (1·03 mol.) were close to the calculated values of 6, 4, and 1 mol., respectively. (5) The specific optical rotation of the sugar and the properties of its crystalline acetate were identical with those of specimens obtained by chemical synthesis [4] (see Table).

Reducing Components of the Laminarin Hydrolysate.—The reducing monosaccharide components were D-glucose (55 g.) and L-fucose (0·55 g.) (see Table). It seems likely that the fucose arose from contamination of the laminarin with fucoidin. No fucose-containing oligosaccharides were found (cf. preceding paper [1]). The disaccharide components were laminaribiose (25 g.), gentiobiose (0·36 g.), and isomaltose (0·09 g.), all three being characterised as crystalline acetyl derivatives (see Table). There were four reducing trisaccharides, namely, laminaritriose (20 g.), 6-*O*-β-laminaribiosylglucose (0·36 g.), 3-*O*-β-gentiobiosylglucose (0·48 g.), and 3-*O*-β-isomaltosylglucose (0·12 g.). The last three substances were identified as follows:

6-O-β-*Laminarobiosylglucose.* (1) Partial acidic hydrolysis gave laminaribiose, gentiobiose, and glucose. (2) Exo-β-glucosidase gave gentiobiose and glucose. (3) The specific optical rotation of the sugar was close to the calculated value and to that of an authentic specimen prepared by chemical synthesis [5] and the crystalline acetyl derivatives of both specimens were identical (see Table).

3-O-β-*Gentiobiosylglucose.* (1) Partial acidic hydrolysis gave gentiobiose, laminaribiose, and glucose. (2) Exo-β-glucosidase liberated laminaribiose and glucose. (3) The specific optical rotation of the sugar was close to that of an authentic specimen [5] and to the calculated value (see Table).

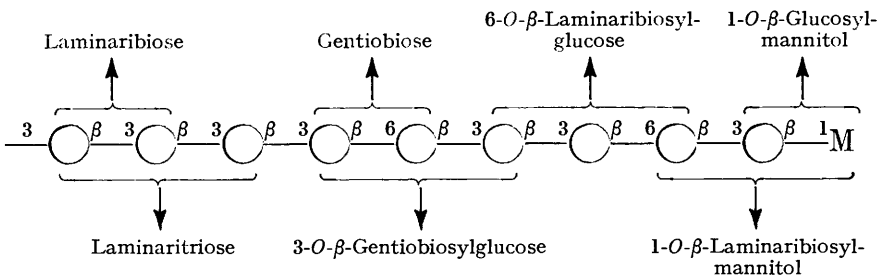
3-O-β-*Isomaltosylglucose.* (1) Partial acid hydrolysis gave glucose, isomaltose, and laminaribiose. (2) Exo-β-glucosidase had no action on the sugar.

The Structure of Insoluble Laminarin.—The foregoing account has shown that fourteen mono-, di-, and tri-saccharide components of the partial acid hydrolysate of laminarin were identified. The question arises whether any of these are artefacts derived from contaminants of the polysaccharide or formed by acid-reversion reactions. There is no doubt as to the origin of the glucose and laminari-biose and -triose, but the fucose is not considered to have any structural significance (see above). The source of the mannitol and the related non-reducing oligosaccharides requires more detailed consideration. It

[4] Peat, Whelan, Lawley, and Evans, *Biochem. J.*, 1955, **61**, x.
[5] Peat, Whelan, and Evans, to be published.

will be recalled that Lindberg [3] found mannitol, 1-*O*-β-glucosylmannitol, and 1 : 6-di-*O*-β-glucosylmannitol in the uncombined state in an extract of *Fucus vesiculosus* but chromatographic examination of the unhydrolysed laminarin failed to reveal the presence of these sugars. If mannitol is an integral part of the laminarin molecule then the partial hydrolysate should contain an oligosaccharide in which both mannitol and 1 : 3-linked β-glucose residues are present. Such a substance was found, namely, 1-*O*-β-laminaribiosylmannitol. In a preliminary communication we reported that this sample of insoluble laminarin contained 1 mole of mannitol to 58 moles of glucose.[6] (The method of estimating the mannitol content of laminarin is described below.) The mannitol present in the 1-*O*-β-glucosylmannitol and the 1-*O*-β-laminaribiosylmannitol represented about 50% and 15%, respectively, of the total mannitol in the amount of laminarin (137 g.) submitted to partial hydrolysis. It is concluded that the reducing ends of at least some of the glucose chains of laminarin are combined glycosidically with the primary hydroxyl groups of D-mannitol. The possibility exists that some of the mannitol residues are incorporated within the polysaccharide chains rather than as terminal units. In such a case, however, it is to be expected that oligosaccharides would be liberated, by partial hydrolysis, in which a mannitol unit is flanked by glucose units. One such fragment, namely, 1 : 6-di-*O*-β-glucosylmannitol, was in fact isolated, but in an amount so small relative to the yield of laminaribiosylmannitol (see Table) that it would be unwise to

Suggested structure of laminarin, showing the origin of the di- and tri-saccharides produced by partial hydrolysis.



Key: ○ = D-glucopyranose unit; M = mannitol
(N.B. Some of the molecules do not contain mannitol.)

ascribe a structural significance to it until more is known about the participation of mannitol in acid-reversion reactions. All that can be said at this stage is that a very large majority of the mannitol residues in laminarin are terminal units.[4] It has been shown that when glucose is heated in acid under the same conditions as for the hydrolysis of insoluble laminarin (0·33N-sulphuric acid for 3 hr. at 100°) about 0·04% is converted into gentiobiose and 0·06% into isomaltose.[7] The yields of these two sugars from insoluble laminarin were 0·26% and 0·06%, respectively, suggesting that while the β-1 : 6-link may be present in laminarin, the α-1 : 6-link is not. Support for the presence of a small proportion of β-1 : 6-links comes from the isolation of significant amounts of 3-β-gentiobiosylglucose and 6-β-laminaribiosylglucose.

The Figure shows diagrammatically a section of a laminarin chain which on partial hydrolysis would yield those of the oligosaccharides isolated which are considered to be true structural entities. This makes laminarin a linear molecules terminated by mannitol and containing mainly β-1 : 3-linked glucose units, interrupted by an occasional β-1 : 6-linkage. We have as yet no information on the possible branching of the molecule. The 1 : 6-bonds could represent points of branching but if this were so, a partial hydrolysate

[6] Peat, Whelan, and Lawley, *Chem. and Ind.*, 1955, 35.
[7] Peat, Whelan, Edwards, and Owen, *J.*, 1958, 586.

might be expected to contain 3 : 6-di-*O*-β-glucosylglucose. We were unable to detect this sugar in the laminarin hydrolysate but such negative evidence cannot be regarded as conclusive.

This representation of laminarin is not intended to imply that all molecules are terminated by mannitol. On the contrary, we have evidence, which will be published later, that samples of insoluble laminarin contain roughly equal numbers of reducing and non-reducing molecules. This is in agreement with a recent report by Unrau and Smith.[8]

The fact that some of the laminarin chains are terminated by mannitol means that they will be completely protected from the erosive actions of both alkali and periodate. Each of these reagents degrades unprotected β-1 : 3-linked glucose polymers stepwise

Physical properties of the partial hydrolysis products of insoluble and soluble laminarin.

Sugar [a]	Source [b]	Yield [c] (g.)	$[\alpha]_D$ in H_2O	β-Acetate m. p. [d]	β-Acetate $[\alpha]_D$ in $CHCl_3$
D-Glucose	I	55	+51·1°	132—133°	—
	S	5·82	+50·9	132—133	+ 3·8°
	A	—	+52·6	135·5	+ 3·8
D-Mannitol	I	0·55	—	122—123	+25·0
	A	—	—	122	+25·0
L-Fucose	I	0·55	—	169	L-Fucose phenylhydrazone
	S	0·39	—	166—167	
	A	—	—	170—173	
Laminaribiose	I	25	+19·1	162	−28·2
	S	2·22	+18·6	162—163	−28·9
	A	—	+18·6	160—161	−28·8
1-*O*-β-Glucosylmannitol	I	2·3	−20·7	88—92	+39·7 [e]
	S	0·37	−20·4	—	—
	A	—	−20·0	88—94	+39·7 [e]
Gentiobiose	I	0·36	+ 8·5	191—192	− 4·8
	S	0·31	+ 9·4	192—193	− 5·0
	A	—	+ 9·6	193	− 5·3
Isomaltose	I	0·09	+121	145—146	+97·5
	S	Not found	—	—	—
	A	—	—	143—144	+97
Laminaritriose	I	20	+ 2·3	120—121	−40·4
	S	2·41	+ 2·0	120—121	−40·1
1-*O*-β-Laminaribiosylmannitol	I	1·1	−24·8	144—145	−18·6
	A	—	−24·3	144—145	−17·9
	C	—	−24·3	—	—
3-*O*-β-Gentiobiosylglucose	I	0·48	− 1·0	—	—
	A	—	− 2·1	—	—
	C	—	− 3·2	—	—
6-*O*-β-Laminaribiosylglucose	I	0·36	− 4	214—215	−27
	A	—	− 6·0	216—217	−27·4
	C	—	− 3·5	—	−24·2

[a] Also isolated from insoluble laminarin were 3-*O*-β-isomaltosylglucose (0·12 g.; $[\alpha]_D^{18°}$ +67° in water), 1 : 6-di-*O*-β-glucosylmannitol (0·04 g.; $[\alpha]_D^{18°}$ −16·5° in water), 1-*O*-β-isomaltosylmannitol (0·01 g.), an isomaltose-containing tetrasaccharide (0·11 g.), and two unidentified sugars (*A* and *B*, see Experimental section). [b] I = Insoluble laminarin, S = soluble laminarin, A = authentic specimen, C = calculated value (see Experimental section). [c] From 180 g. of undried insoluble laminarin containing 137 g. of polyglucose hydrolysed to 55% conversion into glucose and from 26·95 g. of dried soluble laminarin containing 24·6 g. of polyglucose. [d] Mixed m. p. determinations with authentic specimens were carried out wherever possible. No depression was observed. [e] Benzoyl derivative.

from the reducing chain ends. A 1 : 6-linkage in such a chain presents a barrier to erosion, reaction ceasing when the 1 : 6-link is reached by the reagent. It is found in practice that only about half of the glucose residues of insoluble laminarin are eliminated by alkali [8] or periodate [9] and these results have been explained by postulating the existence of 1 : 6-links in the molecule. This interpretation has in turn been used in support of the

[8] Unrau and Smith, *Chem. and Ind.*, 1957, 330.
[9] Corbett and Kenner, *J.*, 1955, 1431.

idea of branching,[10] despite the published evidence [2,4,6] that two types of "blocking" structure are present in laminarin. It is clear that the presence of mannitol units in the laminarin molecule invalidates any conclusions from the alkali or periodate experiments concerning the presence of 1 : 6-links.

The Structure of Soluble Laminarin.—Soluble laminarin has been examined in less detail than the insoluble form. The results were much the same as for insoluble laminarin except that the minor structural features, in the form of mannitol and β-1 : 6-links, were more prominent (see Table). The minor trisaccharides were not examined so that the question of branching remains unresolved.

Experimental

Analytical and General Methods.—Most of the methods are described in the preceding paper [1] and by Peat, Whelan, and Roberts.[12] When $[\alpha]_D$ values of free sugars and laminarin were being measured, the concentrations were determined by acidic hydrolysis to glucose;[13] the concentrations of ester derivatives were measured by weighing. Optical rotations were measured in 4-dm. tubes. M. p.s are uncorrected.

Most paper-chromatographic observations and all preparative paper chromatography was carried out with butan-1-ol–acetic acid–water (4 : 1 : 5, by vol.) as the irrigating solvent. To effect a clear-cut separation of glucose and mannitol the solvents used were ethyl acetate–acetic acid–water (3 : 1 : 1, by vol.) and phenol–water (4 : 1, by weight).

Periodate oxidations were carried out by mixing equal volumes of 0·37M-sodium metaperiodate and sugar solution (0·3—1·4 mg./ml.), and then storing the mixture in the dark at room temperature. Periodate consumption was measured by adding 1 ml. of the digest to a mixture of water (50 ml.), 10% potassium iodide (3 ml.), and 2N-sulphuric acid (2 ml.), the liberated iodine being titrated with 0·033N-sodium thiosulphate, starch glycollate being used as indicator. The titre was compared with that of a sugar-free digest. Formic acid was determined by adding 1 ml. of digest to 0·3 ml. of neutral ethylene glycol. After the sides had been washed with water (5 ml.) the stoppered flask was stored in the dark for 30 min. Neutral 10% potassium iodide (2 ml.) was added and the liberated iodine rapidly titrated with 0·005N-thiosulphate, starch glycollate being used as indicator. A sugar-free digest was similarly titrated. This blank titre was usually nil. Under these conditions oxidation of 1-*O*-β-glucosyl- and 1-*O*-β-laminaribiosyl-mannitol was complete within 2 hr.

The specific optical rotations of some of the trisaccharides were calculated by regarding the sugar as a disaccharide substituted in its non-reducing glucose unit by β-glucose in 1 : 3- or 1 : 6-combination. For example, the molecular rotation and hence $[\alpha]_D$ of 1-*O*-β-laminaribiosyl-mannitol was calculated by adding together the molecular rotation of 1-*O*-β-glucosylmannitol and the molecular rotational increment in the series of laminaridextrins, taken from the results given in Part I.[1] The other molecular rotational increment required was that of the gentiodextrin series of sugars and was taken from the values given by Haq and Whelan.[14]

Insoluble Laminarin

Partial Hydrolysis of Insoluble Laminarin.—Insoluble laminarin, isolated from *Laminaria cloustoni* as by Black, Cornhill, Dewar, and Woodward,[15] was kindly provided by Dr. E. T. Dewar. The vacuum-dried polysaccharide contained 93·1% of polyglucose, and 0·33% of non-volatile matter, and had $[\alpha]_D^{16}$ −10·9° (*c* 0·8 in water). The amorphous triacetate (56% yield, method of Percival and Ross [16]) had $[\alpha]_D^{15}$ −65·6° (*c* 0·45 in chloroform) [Found: Ac, 43·5. Calc. for $(C_{12}H_{16}O_8)_n$: Ac, 44·8%]. Paper-chromatographic analysis of a sample hydrolysed to completion with sulphuric acid [1] showed the presence of a major component with the R_F value of glucose and small amounts of substances corresponding to mannitol and fucose.

[10] Hough and Perry, *Chem. and Ind.*, 1956, 768.
[11] Broatch and Greenwood, *Chem. and Ind.*, 1956, 1015.
[12] Peat, Whelan, and Roberts, *J.*, 1957, 3916.
[13] Pirt and Whelan, *J. Sci. Food Agric.*, 1951, **2**, 224.
[14] Haq and Whelan, *J.*, 1956, 4543.
[15] Black, Cornhill, Dewar, and Woodward, *J. Appl. Chem.*, 1951, **1**, 505.
[16] Whelan, Bailey, and Roberts, *J.*, 1953, 1293.

Two batches of the laminarin were hydrolysed as described previously.[1] Each contained 68·5 g. of polyglucose. The times of hydrolysis at 100° were 165 min. and 170 min., when the percentage apparent conversions into glucose, measured on the neutralised hydrolysates, were 53·4% and 55·3%, respectively. The combined solutions were concentrated to about 1 l., crystallised sodium sulphate being removed from time to time. Paper chromatography showed that glucose, mannitol, fucose, laminaridextrins, and non-reducing oligosaccharides were present. The concentrate was adsorbed on charcoal–Celite (120 × 8·1 cm.; equal weights of B.D.H. "activated charcoal" and Celite no. 535) and the monosaccharide fraction eluted under suction with water. Oligosaccharides were then desorbed by feeding the column from a reservoir initially containing 20 l. of water, the level in which was automatically maintained by a feed of 20% ethanol. After 195 fractions (500 ml. each) had been collected the column was eluted with 50% ethanol until the optical rotation had returned to zero and then with 25% propan-1-ol. The last two fractions weighed 22·5 g. and 24·9 g., respectively, and contained laminaritriose and sugars of higher molecular weight. Fractions 1—195 were examined for optical rotation and the separated sugars were obtained dry as already described.[1, 12, 16]

Monosaccharides.—(a) *Glucose.* Fractions 1—42 (58·5 g.) contained glucose and mannitol. The glucose content was measured by reducing power and the sugar identified by measurement of $[\alpha]_D$ and formation of the crystalline acetyl derivative (89% yield, see Table).

(b) *Mannitol.* To isolate the mannitol it was necessary to remove the large excess of glucose. Attempted removal (in control experiments) of glucose as glucosazone or lead gluconate was unsuccessful and the glucose was therefore converted into the mixed methyl α- and β-glucosides and the charcoal-chromatographic separation of mannitol from the glucosides examined. The desired separation was not however achieved and the glucose was recovered by hydrolysis. Fermentation with yeast was next attempted, control experiments (see later) having shown that the yeast did not introduce any substance which might be mistaken for mannitol.

The mixture (32 g.) was dissolved in water (600 ml.) and treated with fresh baker's yeast (33 g.) for 36 hr. at 30°. After being passed through a Seitz filter, the solution was shown by paper-chromatographic examination to contain mannitol, levoglucosan, isomaltose and/or gentiobiose. These products, apart from the mannitol, were probably formed from glucose during the acidic hydrolysis of the methyl glucosides. They were removed by hydrolysing the solid residue (5·6 g.) in 1·5N-sulphuric acid (2 l.) at 100° for 2 hr. After neutralisation with barium carbonate, the dried residue was extracted with 80% aqueous methanol, the solution evaporated to dryness, and the residue dissolved in water (200 ml.) and again fermented with yeast (10 g.). Only traces of sugars then remained. The syrupy residue (3·7 g.) was acetylated with sodium acetate–acetic anhydride and crystalline D-mannitol hexa-*O*-acetate (1·3 g.) was obtained. This corresponds to 0·55 g. of free mannitol.

(c) *Fucose.* Fractions 43—54 (0·55 g.) contained fucose and a trace of glucose. The The fucose was identified as the phenylhydrazone [17] (51% yield).

Disaccharides.—The sugars are mentioned in order of their elution from the charcoal column.

Fractions 55—61 (0·53 g.) contained isomaltose and the non-reducing disaccharide (1-*O*-β-glucosylmannitol). Fractions 62—68 (2·87 g.) contained the glucosylmannitol, gentiobiose, and traces of laminaribiose and glucose. The two batches were combined and refractionated on charcoal–Celite (57 × 4 cm.) which was eluted with water (2 l.), 4% ethanol (5·6 l.), and then 5% ethanol (2·5 l.), 100 ml. fractions being collected. Products were thereby obtained consisting of isomaltose (0·12 g.) with trace of glucosylmannitol, pure glucosylmannitol (1·37 g.), and fractions containing increasing amounts of gentiobiose mixed with glucosylmannitol (1·1 g.).

(a) *Isomaltose.* The isomaltose fraction was acetylated with sodium acetate—acetic anhydride but the product (179 mg.) did not crystallise. It was fractionated on charcoal–Celite [18] and a crystalline fraction (64 mg.) was obtained having the properties of β-isomaltose octa-acetate. A portion of this acetate was deacetylated [19] in order to measure the $[\alpha]_D$ of the free sugar (see Table).

(b) 1-O-β-D-*Glucopyranosyl*-D-*mannitol.* The chromatographically pure sugar (295 mg.)

[17] Black, Cornhill, Dewar, and Woodward, *J. Sci. Food Agric.*, 1953, **4**, 85.
[18] Morgan and Whelan, to be published.
[19] Bates, "Polarimetry, Saccharimetry, and the Sugars," U.S. Government Printing Office, Washington, 1942, p. 493.

was hydrolysed in 1·5N-sulphuric acid (50 ml.) at 100° until the optical rotation was constant (3 hr.). After neutralisation (barium carbonate) and evaporation the residue (271 mg.) was boiled with 0·25% (w/v) methanolic hydrogen chloride for 90 hr. and then neutralised with silver carbonate. The residue (204 mg.) contained methyl glucoside, mannitol, and a trace of unchanged disaccharide and was fractionated on thick filter paper [12] to yield crystals of methyl α-D-glucoside (53 mg.), m. p. and mixed m. p. 165—166°, $[\alpha]_D^{17}$ +158° (in water). The mannitol residue (76 mg.) was benzoylated [20] to yield crystals (103 mg.) which on recrystallisation from acetone–ethanol gave the hexa-*O*-benzoate, m. p. and mixed m. p. 150—151°, $[\alpha]_D^{16}$ +52·4° (in chloroform). Mannitol and glucose were also identified chromatographically after the disaccharide had been treated with hot 1·5N-sulphuric acid or with almond β-glucosidase.[1]

The disaccharide (137 mg.) was benzoylated [20] and the resulting syrup (605 mg.) deposited crystals (175 mg.) from ethanol which after two further recrystallisations gave 1-*O*-β-D-glucopyranosyl-D-mannitol nona-benzoate. A second specimen was prepared for comparison from an authentic sample of the disaccharide kindly supplied by Dr. Bengt Lindberg, The mixed m. p. showed no depression. All attempts by ourselves and by Dr. Lindberg to obtain a crystalline acetyl derivative have failed.

(c) *Gentiobiose.* Gentiobiose was contained in fractions 55—68 (see above). Fractions 69—75 (9·08 g.) contained mainly laminaribiose with small amounts of glucosylmannitol and gentiobiose. The amounts of gentiobiose in these fractions were estimated by quantitative chromatography on thick filter paper. Because of the similarity of R_F value, quantitative separation of gentiobiose from glucosylmannitol was not attempted. The gentiobiose content of the mixture of the two sugars was estimated by measuring the reducing power with Somogyi reagent [21] which had been calibrated against authentic gentiobiose. For identification some of the gentiobiose in Fractions 69—75 was separated by prolonged irrigation on thick filter paper. The $[\alpha]_D$ of the sugar was measured and its crystalline acetate prepared (65% yield).

(d) *Laminaribiose.* Fractions 76—95 (17·73 g.) contained only laminaribiose. A new derivative of laminaribiose was prepared by treating laminaribiose solution (2·08 g.; 20 ml.) with potassium borohydride solution (526 mg.; 10 ml.) at 20°. The rotation became constant after 3 hr. and 6N-acetic acid was then added until the pH was 4·5. The residue (4·34 g.) obtained by neutralisation with sodium hydroxide and evaporation was treated with sodium acetate–acetic anhydride. The product was *laminaribi-itol nona-acetate*, m. p. 108—109° (from ethanol), $[\alpha]_D^{18}$ −10·8° (*c* 0·53 in chloroform) (Found: C, 49·5; H, 6·1. $C_{30}H_{42}O_{20}$ requires C, 49·9; H, 5·8%).

Trisaccharides.—(a) *Sugars present in trace amounts.* Fractions 96—139 segregated in five batches contained laminaribiose, laminaritriose, and five other sugars. These batches were separately fractionated by chromatography on thick filter paper and examined by partial hydrolysis with sulphuric acid and with emulsin,[1, 12] Three of the components were identified as 3-*O*-β-isomaltosylglucose {movement in butan-1-ol–acetic acid–water relative to laminaribiose (R_L = 0·52), $[\alpha]_D^{18}$ +67° (in water)}, 1-*O*-β-isomaltosylmannitol (R_L = 0·44), and 1 : 6-di-*O*-β-glucosylmannitol {R_L = 0·3; $[\alpha]_D^{18}$ −16·5 (in H_2O); literature [3] $[\alpha]_D^{20}$ −14°}, possibly containing some 1-*O*-β-gentiobiosylmannitol. The other two components (*A*, *B*) which were not identified, gave glucose, laminari-biose and -triose, and higher saccharides on partial acid hydrolysis. Sugar *A* (65 mg.) had R_L = 0·24, $[\alpha]_D^{18}$ +10·7° (*c* 0·07 in water). Sugar *B* (59 mg.) had $[\alpha]_D^{18*}$ −6·9° (*c* 0·09 in water).

(b) 1-O-β-*Laminaribiosylmannitol.* Fractions 140—148 (1·01 g.) consisted mainly of a non-reducing trisaccharide with traces of laminari-biose and -triose. The whole fraction was purified by chromatography on thick filter paper to yield 601 mg. of pure sugar (R_L = 0·44). Reference is made above to the products of its partial acidic and enzymic hydrolysis. The mannitol content of the trisaccharide corresponded to 0·98 mol. The specific optical rotation of the sugar and the properties of its crystalline acetyl derivative (75% yield) are shown in the Table. Measurements were made of the consumption of periodate and the formation of formic acid and of formaldehyde. The sugar solution (6 ml.; 49·4 mg.) was treated with the amount of 1% sodium metaperiodate required for complete oxidation (12·55 ml., 6 mol.) and then stored in the dark for 64 hr. at room temperature; 0·2% aqueous dimedone solution (50 ml.) was then added. After being kept overnight the formaldehyde derivative was filtered off (29·3 mg., 1·03 mol.; m. p. 184—185°, literature, m. p. 188°).

[20] Wolfrom and Gardner, *J. Amer. Chem. Soc.*, 1943, **65**, 750.
[21] Somogyi, *J. Biol. Chem.*, 1945, **160**, 61.

(c) 6-O-β-*Laminaribiosylglucose*. Fractions 149—161 (1·62 g.) contained approximately equal amounts of laminaribiosylmannitol (R_L = 0·46), laminaritriose (R_L = 0·65), and a reducing sugar (R_L = 0·40) migrating between laminari-triose and -tetraose. Laminaritriose was removed by chromatography on thick filter paper but the others were too close in R_F value for clear-cut separation. A successful separation was achieved by converting the reducing sugar (Barker, Bourne, and O'Mant's method [22]) into its methyl furanoside, this having R_L = 1·0. The mixture (699 mg.) was dissolved in 4% (w/w) methanolic hydrogen chloride (35 ml.) by shaking it for 20 min. and set aside at room temperature. The optical rotation became constant (−0·01°; 0·5-dm. tube) after 135 min. and freshly prepared dry silver carbonate (5 g.) was then added. The neutral solution was filtered, the silver residue washed with methanol (3 × 20 ml.), and the combined filtrates evaporated over barium carbonate (freshly washed and dried). Paper chromatography showed the mixture (600 mg.) to be free from reducing sugar and it was fractionated on thick filter paper to yield laminaribiosylmannitol (302 mg.) and the methyl furanoside (104 mg.). The laminaribiosylmannitol was identified by partial acidic and enzymic hydrolysis. The methyl furanoside was hydrolysed in 0·01N-hydrochloric acid (100 ml.) at 44—46°, the reaction being followed by measurement of reducing power, which was constant after 120 hr. After neutralisation (silver carbonate) and isolation in the usual way, the product (48 mg.) contained reducing sugar (R_L = 0·40) and some unchanged glycoside. The reducing sugar was separated on thick filter paper and the product (23 mg.) examined by partial acidic and enzymic hydrolysis (see above). The specific optical rotation was measured and the crystalline acetate prepared.

(d) *Laminaritriose*. Fractions 162—171 (4·33 g.) contained laminaritriose and 6-*O*-β-laminaribiosylglucose. The amount of the latter was estimated by quantitative separation on thick filter paper and hydrolysis to glucose.[13] Fractions 172—195 (9·47 g.) contained only laminaritriose which was characterised as shown in the Table.

(e) 3-O-β-*Gentiobiosylglucose*. The dextrins eluted from the charcoal column by 50% ethanol and 25% propan-1-ol (see above) were refractionated on charcoal–Celite (1 : 1; 145 × 6 cm.) by elution with 22·5% ethanol (9 l.) and then 25% ethanol (4 l.). The first 4 l. of eluate contained no sugar. Subsequently the eluate was collected in 1 l. fractions which were examined by paper chromatography, with the following results: (*a*) glucose and laminari-biose and -triose (0·40 g.), (*b*) laminaritriose (3·89 g.), (*c*) laminaritriose (1·96 g.), (*d*) equal amounts of laminaritriose and a reducing sugar, *X*, migrating between laminari-triose and -tetraose (0·91 g.), (*e*) as (*d*) (0·41 g.), (*f*) as (*d*) with a reducing sugar, *Y*, moving between laminari-triose and -tetraose (0·30 g.), (*g*) laminaritriose, *Y*, and a non-reducing tetrasaccharide (0·61 g.), (*h*) equal amounts of laminaritetraose and non-reducing tetrasaccharide with a trace of *Y* (0·92 g.), (*i*) and (*j*) laminaritetraose (4·00 g.). Fractions (*e*) and (*f*) were further fractionated on thick filter paper to yield *X* (183 mg.), *Y* (106 mg.), and laminaritriose (154 mg.). On partial acidic hydrolysis, *X* gave laminaribiose, gentiobiose, and glucose; *Y* gave glucose, laminaribiose, isomaltose, laminaritriose, and a trisaccharide with the R_F value of 3-*O*-isomaltosylglucose; and laminaritriose gave laminaribiose and glucose. When treated with emulsin, *X* gave laminaribiose and glucose, *Y* was not attacked, and laminaritriose gave laminaribiose and glucose. It was concluded that *X* was 3-*O*-β-gentiobiosylglucose and *Y* was the tetrasaccharide *O*-α-D-glucopyranosyl-(1 ⟶ 6)-*O*-β-D-glucopyranosyl-(1 ⟶ 3)-*O*-β-D-glucopyranosyl-(1 ⟶ 3)-D-glucose. The yield of *X*, characterised by its specific optical rotation, was determined by adding the weight already obtained (183 mg.) to that estimated to be in fraction (*d*) by quantitative chromatography (295 mg.).

Soluble Laminarin

The properties of the sample of soluble laminarin and the details of its hydrolysis and fractionation are described in the preceding paper.[1] Glucose and fucose were isolated and characterised; mannitol was detected by paper chromatography. Glucosylmannitol, gentiobiose, laminaribiose, and laminaritriose were also characterised (see Table).

Estimation of Mannitol in Laminarin.

To estimate mannitol advantage was taken of the large specific optical rotation which mannitol exhibits in the presence of acid ammonium paramolybdate ($[\alpha]_{5461}^{20}$ + 169°; ref. 23).

[22] Barker, Bourne, and O'Mant, *Chem. and Ind.*, 1955, 425.
[23] Frèrejacque, *Compt. rend.*, 1935, **200**, 1410; 1939, **208**. 1123.

In the absence of molybdate, $[\alpha]_D$ is only $-0{\cdot}24°$. The intention was to hydrolyse laminarin with acid, to remove glucose by fermentation with baker's yeast, and then to measure the optical rotation. The stages in the development of the method were as follows:

(i) It was shown that a direct proportionality existed between α_D and concentration of D-mannitol in the range 0—0·19% mannitol in the presence of 1% ammonium paramolybdate and 0·1N-sulphuric acid. The measured $[\alpha]_D^{17}$ of mannitol–molybdate was $+139{\cdot}3°$.

(ii) Glucose (10 g.) in water (200 ml.) was incubated with baker's yeast (11 g.) for 19 hr. at 30° in the presence and absence of mannitol (1 g.). The digests were passed through a Seitz filter and examined by paper chromatography. Glucose, but not mannitol, had completely disappeared and the solutions had no detectable optical activity.

(iii) Mannitol (1 g.) was incubated with a suspension of yeast (10 g.) in water (200 ml.) at 30°. At intervals filtered portions (5 ml.) were diluted to 25 ml. with water only or with, in addition, 5% molybdate (5 ml.) and 5N-sulphuric acid (0·5 ml.) to give the 1% final concentration of molybdate and 0·1N concentration of acid used in all experiments. A yeast suspension free from mannitol was treated in the same way. Only the mannitol–molybdate solution showed optical activity. This was constant during the period 0—48 hr. but fell progessively thereafter, being 93·9%, 80·4%, and 54·7% of its initial value after 71, 95, and 119 hr. In subsequent experiments, therefore, the yeast treatment was continued for not more than 36 hr.

(iv) Four digests containing mannitol (24—240 mg.), glucose (2 g.), yeast (2 g.), and water to 43 ml. were incubated at 30° for 36 hr. in 50 ml. graduated cylinders plugged with cotton wool. After addition of water to 43 ml. to adjust for slight evaporation, the digests were filtered and 5—10 ml. portions diluted to 25 ml. with molybdate and acid or with water. The molybdate solutions were optically active and the recoveries of mannitol corresponded to 104·2% (from 24 mg. of mannitol), 100·9% (72 mg.), 99·5% (120 mg.), 100·7% (240 mg.).

(a) *Insoluble Laminarin.*—The sample examined was that described earlier in this paper. Two vacuum-dried samples (*A*, 1·9476 g.; *B*, 1·9783 g.) were each dissolved in water (200 ml.), and 36N-sulphuric acid (21·5 ml.) and water to 500 ml. were added. After being heated at 100° for 3 hr. the cooled solutions were neutralised with barium carbonate (90 g.) overnight and the filtrate and water washings evaporated to dryness. Aqueous solutions of the residues were filtered, made up to 100 ml. with washings, and incubated with baker's yeast (2 g.) for 36 hr. at 30°. The yeast was removed on a Seitz filter and washed, the filtrates evaporated, and the residues each fractionated on thick filter paper to remove unfermentable reversion products and fucose. The mannitol was extracted with water (3 × 500 ml.) and the extracts evaporated to 20 ml., filtered, and diluted to 50 ml. One portion (15 ml.) was diluted to 25 ml. with water and two further 15-ml. portions with 5% molybdate and 5N-sulphuric acid such that the final concentrations of these reagents were in one case 1% and 0·1N and in the other 1·6% and 0·2N respectively. The water solutions were optically inactive; the molybdate solutions had α_D $+0{\cdot}216°$, $+0{\cdot}213°$ (Sample *A*), and $+0{\cdot}213°$, $+0{\cdot}217°$ (Sample *B*), respectively. These values, taken with the measured polyglucose content (93·1%, see above), correspond to molar ratios of glucose : mannitol of 57·5 : 1 and 58·4 : 1, respectively.

(b) *Soluble laminarin.* Samples of the specimen of soluble laminarin described in Part I [1] (1·8453 g. and 1·8418 g.) were treated as for insoluble laminarin. The glucose : mannitol ratios found were 37·1 : 1 and 37·9 : 1, respectively.

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