663. *The Constitution of Laminarin. Part III.* The Fine Structure of Insoluble Laminarin.*

By F. B. Anderson, E. L. Hirst, D. J. Manners, and A. G. Ross.

Insoluble laminarin, a mixture of glucans isolated from *Laminaria cloustoni*, has been investigated by periodate oxidation and methylation. The presence of mannitol (2%) has been confirmed, and approximately half the molecules are terminated by a mannitol residue. Some of the molecules have a low degree of branching, and the constituent chains appear to be interlinked by β-1 : 6-glucosidic linkages. Many of the branched molecules contain mannitol.

The polysaccharide material isolated after the reduction of laminarin with potassium borohydride, and after treatment with lime-water, has also been examined; their structures support the above conclusions on the fine structure of laminarin.

The molecular structure of laminarin, a mixture of glucans which form a reserve carbohydrate in various species of brown marine algae (Phaeophyceae), has been the subject of several recent investigations. The material exists in two forms which differ in solubility in cold water. A "soluble" form is present mainly in *Laminaria digitata*, whilst "insoluble" laminarin has been isolated from the fronds of a number of species of Laminariaceae. The present paper is concerned only with the laminarin from *L. cloustoni*.

Methylation studies carried out by Barry [1] showed that insoluble laminarin was composed of β-1 : 3-linked D-glucopyranose residues, whilst successive oxidation with periodate and bromine indicated [2] an apparent chain length of sixteen glucose residues. However, a later investigation (Part I [3]) suggested that laminarin was composed of about twenty β-1 : 3-linked glucose residues, although the reducing power was unexpectedly low. It was concluded [3] that a proportion of the reducing glucose groups were in some way modified. A similar investigation of soluble laminarin from *L. digitata* was described in Part II [4] and will be considered in detail in a later communication.

An important development in laminarin chemistry was the discovery that mannitol (*ca.* 2%) was a constituent residue.[5] After partial acid hydrolysis of laminarin, Peat, Whelan, and Lawley separated from the mixed saccharides small quantities of mannitol, 1-*O*-β-D-glucosylmannitol and 1-*O*-laminaribiosylmannitol in addition to much larger amounts of glucose, laminaribiose, and higher laminarisaccharides. This finding, together with other evidence, showed that a *proportion* of the molecules were terminated by a mannitol residue linked through one of the two primary alcohol groups. A further discovery, which suggested that laminarin contained a few β-1 : 6-glucosidic linkages, was the isolation of a small quantity (0·26%) of gentiobiose, together with two isomeric trisaccharides, 6-*O*-β-laminaribiosylglucose and 3-*O*-β-gentiobiosylglucose. Since Peat and his co-workers considered that positive evidence for branching was not available (a significant quantity of 3 : 6-di-*O*-β-glucosylglucose could not be isolated), they suggested that one of the possible structures for laminarin was "a linear molecule of β-glucose residues in which repeating sequences of 1 : 3-links are occasionally interrupted by a 1 : 6-linkage." We are indebted to Professor Peat and Dr. Whelan who kindly allowed us to see two papers [5] before publication.

The present communication describes further methylation and periodate oxidation

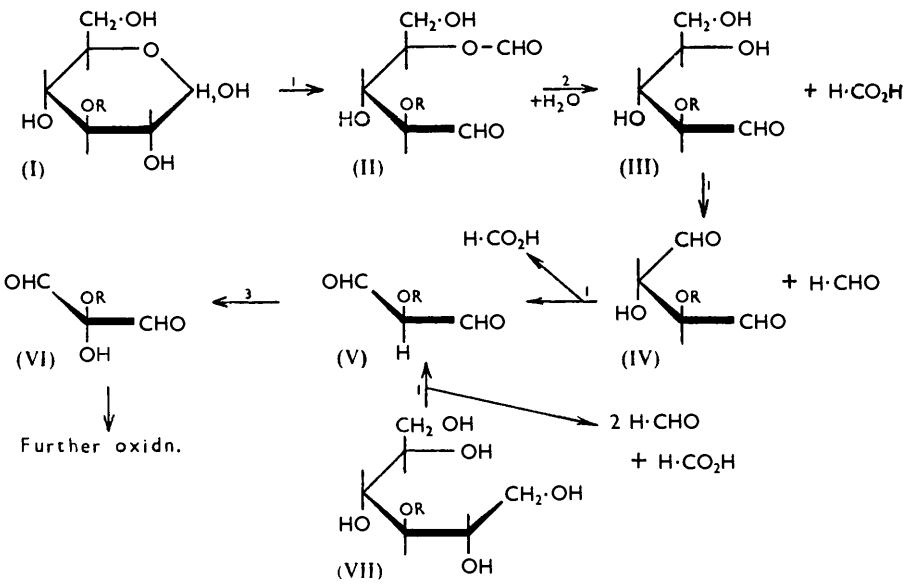
* Part I, *J.*, 1950, 3494; Part II, *J.*, 1951, 720.

[1] Barry, *Sci. Proc. Roy. Dublin Soc.*, 1939, **22**, 59.
[2] Barry, *J.*, 1942, 578.
[3] Connell, Hirst, and Percival, *J.*, 1950, 3494.
[4] Percival and Ross, *J.*, 1951, 720.
[5] Peat, Whelan, and Lawley, *Chem. and Ind.*, 1955, 35; Peat, Whelan, Lawley, and Evans, *Biochem. J.*, 1955, **61**, x; Peat, Whelan, and Lawley, *Proc. Chem. Soc.*, 1957, 340; *J.*, 1958, 724. 729.

studies of insoluble laminarin. Degradation of laminarin by lime-water has also been examined. A preliminary account of part of this work has appeared elsewhere.[6] The following abbreviations are here used: average chain length ($\overline{CL}$) denotes the average number of residues per non-reducing glucose end-group; degree of polymerisation ($\overline{DP}$) refers to the average number of residues per molecule. Chains terminated at the reducing end by glucose or mannitol are described as G-chains or M-chains, respectively.

Isolation and Properties of Laminarin.—The laminarin was one sample, prepared by Dr. W. A. P. Black. It was extracted from the seaweed at 55° with dilute hydrochloric acid (pH 3·4) in 1 hr. and when purified had a glucose content of 94%, $[\alpha]_D$ −9° in H_2O, +9° in N-sodium hydroxide, and contained one reducing group per 47 residues (hypoiodite oxidation). (The insoluble laminarin examined in Parts I[3] and II[4] had reducing powers corresponding to one reducing group per 40 and 45 residues respectively.)

A partial acid hydrolysate was shown by paper chromatography to contain glucose, laminaribiose, laminaritriose, and unidentified sugars with $R_{Glucose}$ values of 0·45 (non-reducing), 0·34, 0·24, and 0·16. A complete acid hydrolysate contained mannitol. Since D-glucose and mannitol have similar R_G values in the usual paper chromatographic solvents, two different experiments were carried out. In the first, the glucose was converted into the less mobile D-gluconic acid by addition of D-glucose oxidase[7] to the neutralised hydrolysate. A non-reducing carbohydrate with the R_G value of mannitol was then revealed.



The presence of mannitol (and a non-reducing disaccharide) has also been shown by using a special solvent (ethyl methyl ketone–acetic acid–water, saturated with boric acid) kindly communicated by Dr. W. R. Rees.

Periodate Oxidation Studies.—Periodate oxidation[8] of a 3-*O*-substituted glucose (I) may involve at least three reactions: (1) normal Malapradian oxidation of α-glycol groups, (2) slow hydrolysis of a formyl ester (II), followed by further Malapradian oxidation, and (3) oxidation of an activated hydrogen atom in a structure (V) of malondialdehyde type. In contrast, periodate oxidation of 3- or 6-*O*-substituted hexitols (VII and VIII) requires

[6] Anderson, Hirst, and Manners, *Chem. and Ind.*, 1957, 1178.
[7] Keilin and Hartree, *Biochem. J.*, 1948, **42**, 230.
[8] For a review, see Bobbit, *Adv. Carbohydrate Chem.*, 1956, **11**, 1.

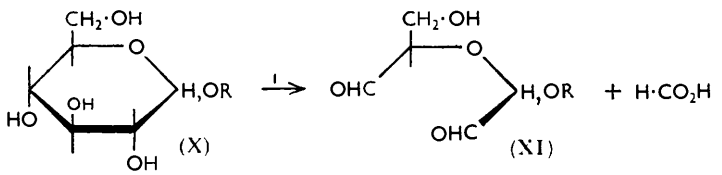
only normal Malapradian-type reactions to yield respectively the malondialdehyde derivative (V) or the substituted acetaldehyde derivative (IX):

$$\underset{\text{(VIII)}}{RO\cdot CH_2\cdot[CH(OH)]_4\cdot CH_2\cdot OH} \xrightarrow{I} \underset{\text{(IX)}}{RO\cdot CH_2\cdot CHO} + 3H\cdot CO_2H + H\cdot CHO$$

Partial acid hydrolysis [5] and methylation [1,3] indicate that in laminarin the chains are terminated at the potential reducing end by either a reducing glucose residue linked at $C_{(3)}$ or a mannitol group, as represented in formulæ (I) and (VIII) respectively (where R is a chain of β-1 : 3-linked glucose residues).

Oxidation of non-reducing end-groups (X) also proceeds without a formyl-ester intermediate, to give the stable dialdehyde (XI).

The relative rates of these reactions have been controlled by using three oxidation conditions: (*a*) Initial primary oxidation (I ⟶ II, VII ⟶ V, VIII ⟶ IX, and X ⟶ XI) by sodium metaperiodate at 2°, whereby the rate of hydrolysis of formyl esters is greatly reduced,[9] (*b*) total primary oxidation (I ⟶ V, and normal oxidation of VII, VIII, and X) with potassium metaperiodate [10] or a limited excess of sodium metaperiodate [11] at room temperature, and (*c*) sodium metaperiodate buffered at pH 8 at room temperature, enabling reactions (1), (2), and (3) to take place.[12]



Control experiments have shown that the initial primary oxidation of laminaribiose (giving structures II and XI) required some 14 hr., and that formaldehyde production from this sugar, or from 3-*O*-methyl-D-glucose, was not observed until after 3 days' oxidation. In contrast, the oxidation of laminarin under similar conditions gave formaldehyde within 1 hr. This observation is not only consistent with the presence of terminal-mannitol residues, but also provides a method of estimation. The initial yield * of formaldehyde (0·02 mol.) is equivalent to the presence of 2% of mannitol in the laminarin.

Measurements of the formaldehyde liberated during the total primary oxidation of laminarin and the corresponding alcohol (laminaritol) have been used to determine the number average $\overline{DP}$ and the proportion of M-chains. This oxidation (with a limited excess of sodium metaperiodate at room temperature) gave 0·041 mol. of formaldehyde from laminarin. Since the oxidation of either G- or M-chains gives rise to one mol. of formaldehyde per chain, this value corresponds to a $\overline{DP}$ of 24. After reduction of laminarin with potassium borohydride (p. 3237) the laminaritol so produced yielded 0·063 mol. of formaldehyde on similar oxidation. It follows that 0·022 mol. of formaldehyde arises from the G-chains in laminarin, and that the remaining 0·019 mol. is liberated from M-chains. Calculations from these data show that the laminarin sample of $\overline{DP}$ 24 contains (*a*) 46% of M-chains, (*b*) 1·9% of mannitol, and (*c*) one reducing group per 45 glucose residues.

During the oxidation of laminarin at pH 8, only the residues (VIII) and (X) of the M-chains will be attacked, whilst linear G-chains will be degraded in stepwise fashion with the production of one mol. of formaldehyde per glucose residue. This oxidation process is

* The products liberated on periodate oxidation are normally reported as mol. per anhydrohexose residue.

[9] Meyer and Rathgeb, *Helv. Chim. Acta*, 1948, **31**, 1540; 1949, **32**, 1102.
[10] Halsall, Hirst, and Jones, *J.*, 1947, 1427.
[11] Manners and Archibald, *J.*, 1957, 2205.
[12] Hough and Perry, *Chem. and Ind.*, 1956, 768.

inhibited by 1 : 6-linkages since these, like the mannitol residue, give rise to stable acetaldehyde derivatives (IX).[12] Our laminarin sample gave 0·52 mol. of formaldehyde, a value similar to that reported by Hough and Perry.[12] This result is in good agreement with that expected for a laminarin containing approximately equal numbers of M- and G-chains, many of the latter being linear.

The $\overline{CL}$ of laminarin can be deduced from the amount of formic acid produced during the initial primary oxidation. The observed figure, after 6 hours' oxidation with potassium metaperiodate at room temperature, or 24 hours' oxidation with sodium metaperiodate at 2° (see p. 3242), is 0·10 mol., *i.e.*, 10 mol. of formic acid per 100 hexose residues. Since each mannitol residue gives rise to 3 mol. of formic acid, 5·7 mol. will originate from these, and the remainder (4·3 mol.) must arise from non-reducing end-groups. The proportion of these is therefore one per 23 glucose residues. In view of later evidence of heterogeneity, this numerical agreement between the $\overline{DP}$ and $\overline{CL}$ values is considered to be fortuitous, and not an indication of a linear structure.

The proportion of free reducing groups in the polysaccharide may be estimated from the rate of production of formic acid. As stated above, the initial primary oxidation yields 0·10 mol. whereas the total oxidation [after the hydrolysis of formyl ester groups (II) which requires 8—10 days] gives 0·15 mol. Since 0·05 mol. of formic acid is liberated from reducing glucose end-groups, the proportion of these is one per 40 glucose residues (cf. one per 45—47 by other methods).

Methylation Studies.—Laminarin was methylated with dimethyl sulphate and sodium hydroxide, and a chloroform solution of the methylated polysaccharide (OMe, 44·0%) was fractionally precipitated with light petroleum (b. p. 40—60°). The precipitate amounted to 78% of the methylated polysaccharide; the material which was soluble in light petroleum has not been examined further.

Hydrolysis of insoluble methylated laminarin gave 2 : 3 : 4 : 6-tetra- (4·4%), 2 : 4 : 6-tri- (84·6%), 4 : 6-di- (1·9%), unidentified di- (7·2%), and mono-*O*-methyl-D-glucose (1·9%). The proportion of tetra-*O*-methylglucose is equivalent to a $\overline{CL}$ value of 23 (not 20 as stated in ref. 13). No tri-*O*-methylglucose other than the 2 : 4 : 6-isomer could be detected.

The molecular weights of the whole and of insoluble methylated laminarin, as determined by isothermal distillation,[13] were respectively 1900 and 12,000, equivalent to $\overline{DP}$ values of 9 and 58. It follows that the material (22%) soluble in light petroleum had a molecular weight of *ca.* 500 ($\overline{DP}$ 2—3).

Although the measurement of molecular weights in the range 2000—20,000 is extremely difficult, the isothermal distillation measurements (which in this range are accurate to within 10%) show that the $\overline{DP}$ of methylated laminarin is much larger than the $\overline{CL}$, and a number of the molecules must therefore contain a small number of branch points. On the average, two branch points per molecule appear to be present. Evidence on the nature of the interchain linkage has not been obtained from the methylation analysis since undermethylation of the polysaccharide and hydrolytic demethylation of 2 : 4 : 6-tri-*O*-methylglucose [3,4] both give rise to di-*O*-methylglucose. However, the presence of oligosaccharides containing β-1 : 6-glucosidic linkages in partial hydrolysates of laminarin [5] suggests that these are, in fact, present as interchain linkages. These would number only 2—3% of the total glucosidic linkages, in agreement with the small observed yield of gentiobiose. The failure to isolate 3 : 6-di-*O*-β-glucosylglucose from a partial hydrolysate is not inconsistent with this view. In analogous experiments with other branched polysaccharides, hydrolysis of glycosidic linkages adjacent to interchain linkages does not appear to be random. Thus, the major trisaccharide containing an interchain linkage in a partial hydrolysate of glycogen or amylopectin is panose; [14] significant quantities of 4 : 6-di-*O*-α-glucosylglucose have not been isolated.

[13] Broatch and Greenwood, *Chem. and Ind.*, 1956, 1015.

[14] Peat, Whelan, and Edwards, *J.*, 1955, 355; Thompson and Wolfrom, *J. Amer. Chem. Soc.*, 1951, **73**, 5849.

Degradation of Laminarin with Lime-water.—The susceptibility of reducing glucosaccharides and, in particular, laminarin to degradation by aqueous alkali is well known (see, for example, Part II [4]). Recent studies by Corbett and Kenner [15] showed that insoluble laminarin, on prolonged treatment with oxygen-free lime-water at 25°, is degraded in stepwise fashion from the reducing group to give D-glucometasaccharinic acid in 40—50% yield. Termination of the reaction was ascribed " to some inhibitive variation in chain structure " and Corbett and Kenner pointed out that degradation by lime-water would cease when 1 : 6-linkages were encountered in the molecule. It is now clear, however, that the degradative action of lime-water must be confined to the G-chains, and in laminarin samples containing an appreciable number of M-chains incomplete conversion into D-glucometasaccharinic acid is to be expected.

We carried out similar experiments, but at 60°, and subjected the residual polysaccharide to methylation analysis. Laminarin was treated with oxygen-free lime-water for 23 hr. at 60°. Unattacked polysaccharide which separated was collected (46% recovery). Re-treatment of this material with lime-water did not cause any appreciable degradation, so that more than 50% of the laminarin had been transformed into soluble products. The lime-treated laminarin had $[\alpha]_D^{20}$ −7° (in H_2O), only a very slight reducing power, and contained mannitol. On partial hydrolysis with oxalic acid, a series of oligosaccharides similar to those in the laminarin hydrolysate (p. 3234) were obtained.

Methylated lime-treated laminarin was prepared (OMe, 44·1%) and fractionated with light petroleum. It proved to be more homogeneous than the methyl ether of laminarin as 88% of the material was insoluble in light petroleum. This fraction, which had a molecular weight of 13,600 by isothermal distillation [13] (equivalent to $\overline{DP}$ 65), will be referred to as " methylated lime-treated laminarin." Since the whole methylated sample had a molecular weight of 2500, the material (12%) soluble in light petroleum consisted of methylated saccharides of $\overline{DP}$ *ca.* 2.

An acid hydrolysate of " methylated lime-treated laminarin " (see above) contained the following sugars: 2 : 3 : 4 : 6-tetra- (5·2%), 2 : 4 : 6-tri- (84·4%), 4 : 6-di- (1·5%), unidentified * di- (7·2%) and mono-*O*-methyl-D-glucose (1·8%). These proportions are generally similar to those present in the hydrolysate of methylated laminarin (p. 3236). The " methylated lime-treated laminarin," like methylated laminarin, thus contains molecules with a low degree of branching, namely, an average of two branch points per molecule.

The sedimentation of lime-treated laminarin in the ultracentrifuge has been compared, by Broatch and Greenwood,[13] with that of the original polysaccharide. The insoluble laminarin was extremely heterogeneous and had a sedimentation constant of $0·5 \times 10^{-13}$ c.g.s. units, whereas the lime-treated material was more homogeneous and had a much larger sedimentation constant ($1·0 \times 10^{-13}$ c.g.s. units). These results indicate that the lime-water treatment has preferentially degraded the polysaccharide material of low molecular weight. This degradation is confined to G-chains, and many of the molecules of higher molecular weight (branched) must therefore be terminated by mannitol residues.

Preparation and Methylation of Laminaritol.—Free reducing groups in a polysaccharide can be reduced to the corresponding alcohol residue. The conversion of laminarin into laminaritol (with G-chains terminated as in VII) was first reported by Abdel-Akher, Hamilton, and Smith [16] who used sodium borohydride as reducing agent. With potassium borohydride, we have prepared laminaritol which was virtually non-reducing, with $[\alpha]_D$ −8° (in H_2O) and a glucose content of 94%.

* *Added May 10th, 1958*: This di-*O*-methyl sugar has now been identified as the 2 : 4-isomer since it could be differentiated from 2 : 6- and 4 : 6-di-*O*-methylglucose by paper electrophoresis, and on periodate oxidation formaldehyde was liberated. This finding is in accord with, but not proof of, a branched structure.

[15] Corbett and Kenner, *J.*, 1955, 1431.
[16] Abdel-Akher, Hamilton, and Smith, *J. Amer. Chem. Soc.*, 1951, **73**, 4691.

If our previous deductions on the structure of laminarin and its periodate oxidation are correct, then laminaritol will consist of a mixture of M-chains and sorbitol-terminated chains (S-chains). The laminaritol should therefore be resistant to lime-water, should be overoxidised to virtually the same extent as laminarin, and on periodate oxidation the rate of release of formaldehyde and formic acid should be increased.

The above conclusions have been verified experimentally. Lime-water at 60° had no appreciable action on laminaritol. On overoxidation with periodate, 0·55 mol. of formaldehyde was produced. On oxidation at pH 5, the formaldehyde production from laminaritol before and after lime-water treatment was unaltered. The maximum constant yield after only 6 hours' oxidation was 0·063 mol., equivalent to a $\overline{DP}$ of 24. Further, the amount of formic acid (*ca.* 0·13 mol.) released on total primary oxidation was, as expected, less than from laminarin, and the rate of production was increased.

Methylation of laminaritol gave a polysaccharide methyl ether with OMe 43·3%, and only 6% of this failed to be precipitated when light petroleum was added to a chloroform solution. The precipitated methylated laminaritol had a number-average molecular weight of 3800 ($\overline{DP}$ 19) from isothermal-distillation measurements.[17] This fractionation differed from that of methylated laminarin in that material of higher molecular weight ($\overline{DP}$ *ca.* 60) was not selectively precipitated.

The Molecular Structure of Laminarin.—From the evidence cited above we conclude that " laminarin " is heterogeneous with respect both to molecular weight and to chemical structure. The conditions used for its extraction (pH 3·4 and 55°) do not cause marked degradation (see p. 3239) and it seems probable that " native " laminarin is also heterogeneous. In agreement with Peat and his co-workers, we find that insoluble laminarin contains *ca.* 2% of mannitol, and that approximately one-half of the molecules are terminated by mannitol. Unrau and Smith [18] have concluded, independently, that 30% of M-chains were present in an unspecified sample of laminarin. The heterogeneous nature of the latter was also shown [18] by electrophoresis on glass-fibre paper. In addition, although the average $\overline{DP}$ of our laminarin is *ca.* 24, some of the molecules (78% *by weight*) have a $\overline{DP}$ of *ca.* 60 and are slightly branched with β-1 : 6-inter-chain linkages. The alternative suggestion [5] that the β-1 : 6-glucosidic linkages are located in non-terminal positions in a linear chain of β-1 : 3-linked glucose residues seems less likely since this structure, on methylation analysis, would yield a small quantity of 2 : 3 : 4-tri-*O*-methyl-glucose. This sugar has not been detected. Further, the number of triol groups in laminarin is the same by both methylation and periodate oxidation analysis, in agreement with a branched structure. In a linear-type structure, the number of triol groups detected by periodate would exceed that found from methylation.

The methylation analysis of laminarin is complicated, in part, by the presence of alkali-sensitive reducing groups and, in contrast to laminaritol, laminarin is partly degraded on methylation, the number-average molecular weight decreasing from *ca.* 4000 to *ca.* 1900. Similar observations have been made by Friedlaender, Cook, and Martin [19] who found partly methylated laminarin with OMe 2·7 and 6·9% had molecular weights (weight-average values from sedimentation-diffusion measurements) of 3700 and 2900 respectively. This degradation is presumably limited to G-chains, which are partly converted into methylated oligosaccharides with $\overline{DP}$ 2—3. (In our previous study,[3] these oligosaccharides would be separated from the methylated laminarin during the purification of the latter by dialysis.) However, the residual laminarin (78%) has an average chain length of 23, and a proportion of these molecules are slightly branched. It is of interest that many of these branched molecules are resistant to lime-water at 60° and are therefore terminated by mannitol residues.

[17] Broatch, Ph.D. Thesis, Edinburgh, 1956.
[18] Unrau and Smith, *Chem. and Ind.*, 1957, 330; see also Lewis and Smith, *J. Amer. Chem. Soc.*, 1957, **79**, 3929.
[19] Friedlaender, Cook, and Martin, *Biochim. Biophys. Acta*, 1954, **14**, 136.

Experimental

Analytical Methods.—(a) *Paper chromatography.* The following solvents were used for the separation of glucose and mannitol: (*a*) phenol–water (72 : 28) with a silver nitrate identification spray; (*b*) ethyl methyl ketone–acetic acid–water, saturated with boric acid (9 : 1 : 1, v/v) [20] with a periodate–benzidine spray reagent.[21] The solvents used for the chromatography of oligosaccharides and methylated sugars have been described previously.[22]

(b) *Reducing power.* Somogyi's alkaline copper reagent [23] or Hagedorn and Jensen's potassium ferricyanide method [24] was used, with glucose and laminaribiose as standard sugars. Reducing power (R.P.) values are expressed as 100/No. of glucose residues per apparent reducing group (against laminaribiose standard).

(c) *Periodate oxidation.* The consumption of periodate and the production of formic acid were determined as described by Manners and Archibald.[11] Formaldehyde was estimated qualitatively with a chromotropic acid reagent [25] and quantitatively by the method of Hough, Powell, and Woods.[26]

Preparation and Properties of Insoluble Laminarin.—The laminarin was prepared by Dr. W. A. P. Black, Institute of Seaweed Research, Inveresk, as follows: Fresh *L. cloustoni* fronds, collected at Campbeltown (November, 1954), were minced ($\frac{1}{4}$″ mesh) and extracted for 1 hr. at 55—60° with ten parts (by wt.) of dilute hydrochloric acid solution (pH 3·4). After centrifugation, the weed residue was washed with water, and the combined extract and washings were set aside for 48 hr. The polysaccharide deposited was collected and washed with alcohol and ether. The yield represented 43% of the laminarin present in the fronds. The laminarin, purified by recrystallisation from hot water, had $[\alpha]_D$ −9° (*c* 1·5 in H_2O), +9° (*c* 2·7 in N-NaOH) [adsorbed alcohol,[27] 0·20; ash content, 0·45; glucose content (by cuprimetric titration), 94%; R.P. values, 2·5 (Somogyi), 5·9 (potassium ferricyanide)]. Hypoiodite oxidation indicated the presence of one reducing group per 47 glucose residues.

Stability of Laminarin at pH 3·4 *and* 55°.—Laminarin (1 g.) was heated at 55° with dilute hydrochloric acid solution (pH 3·4; 100 ml.). Samples were removed after 0, 30, and 60 min., cooled, and neutralised, and the reducing powers of 5 ml. portions were determined. No increase in reducing power was observed. The residual solution was cooled, neutralised, and evaporated to dryness. Oligosaccharides could not then be detected by paper chromatography.

Partial Acid Hydrolysis of Laminarin.—Laminarin (1 g.) was heated at 100° with 0·1N-oxalic acid (40 ml.) for 5·5 hr., cooled, and neutralised with calcium carbonate, and the filtrate, after concentration, was examined by paper chromatography. The hydrolysate contained glucose, laminaribiose, laminaritriose (by comparison of $R_{Glucose}$ values with those of authentic samples), R_G 0·45 (non-reducing), R_G 0·34, 0·24, and 0·16 (reducing). Laminaritetraose would have an R_G value of 0·24 under these conditions. In addition, smaller amounts of non-reducing oligosaccharides were present.

Complete Acid Hydrolysis of Laminarin.—Laminarin (1 g.) was hydrolysed with N-sulphuric acid (100 ml.) for 7·5 hr. at 100°, and the solution cooled, neutralised with barium carbonate, filtered, and evaporated to a syrup. The presence of glucose was shown by paper chromatography, with both aniline oxalate and silver nitrate sprays. Part of the syrup (0·1 g.) was incubated with glucose oxidase solution (5 ml.) at pH 7·0 for 40 hr. The residual sugar with R_G *ca.* 1 reacted with silver nitrate but not with aniline oxalate. Chromatography in phenol–water revealed a non-reducing carbohydrate with the R_G value of mannitol.

In later experiments, neutralised hydrolysates were chromatographed in the solvent containing boric acid. Authentic samples of mannitol and sorbitol had R_G 2·8 and 3·3 respectively. The hydrolysate contained non-reducing carbohydrates with R_G 2·8 and 1·6.

Methylation Analysis of Laminarin.—The polysaccharide (15 g.) was methylated four times at room temperature under nitrogen, with dimethyl sulphate and sodium hydroxide solution. The methylated polysaccharide was extracted with chloroform, and the extract washed with

[20] Rees and Reynolds, *Nature*, 1958, **181**, 767.
[21] Cifonelli and Smith, *Analyt. Chem.*, 1954, **26**, 1132.
[22] Chanda, Hirst, and Manners, *J.*, 1957, 1951.
[23] Somogyi, *J. Biol. Chem.*, 1952, **195**, 19.
[24] Hagedorn and Jensen, *Biochem. Z.*, 1923, **135**, 46.
[25] Mitchell and Percival, *J.*, 1954, 1423.
[26] Hough, Powell, and Woods, *J.*, 1956, 4799.
[27] Newman, *J. Pharmacol.*, 1936, **56**, 278.

water, dried, and concentrated (yield, 18·5 g., 98%) (Found: ash, 1·0; OMe, 44·0. Calc. for tri-*O*-methyl-laminarin: OMe, 45·6%).

Methylated laminarin (16·9 g.) was dissolved in chloroform (100 ml.), and light petroleum (b. p. 40—60°) added slowly until precipitation occurred. The material which was precipitated between 91 and 92·5% v/v light petroleum content was collected, washed, and dried (12·8 g., 78%; $[\alpha]_D$ −5·7° at *c* 1·1 in $CHCl_3$). No further polysaccharide was precipitated when the concentration of light petroleum was increased to 95%.

By isothermal distillation,[13] the original and the precipitated sample of methylated laminarin had molecular weights of 1900 and 12,000 respectively. An acid hydrolysate of the original methylated laminarin contained tetra-, tri-, and di-*O*-methylglucose (paper chromatography).

Precipitated methylated laminarin (5 g.) was hydrolysed with methanolic 4% hydrogen chloride (160 ml.) in a sealed tube at 100° for 6·5 hr. After neutralisation with silver carbonate, the filtrate was concentrated and hydrolysed with boiling aqueous N-hydrochloric acid (300 ml.) for 14 hr. The neutralised, concentrated hydrolysate was extracted with acetone and on evaporation 5·3 g. of methylated glucose derivatives were obtained.

The mixed sugars were chromatographed in butan-1-ol (15 ml.) on cellulose [28] (50 × 3 cm.), with light petroleum (b. p. 100—120°)–butan-1-ol saturated with water (7 : 3) as eluant. Three fractions were collected, comprising 4·85 g. of methylated sugars. Elution of the column with light petroleum–butan-1-ol (1 : 1) and then water yielded two further fractions (0·59 g.).

Fraction 1 (6 mg.) was not examined.

Fraction 2 (0·246 g.) contained 2 : 3 : 4 : 6-tetra-*O*-methylglucose (94%, by hypoiodite oxidation) and was homogeneous on paper chromatography; recrystallised from light petroleum (b. p. 40—60°) it had m. p. 85—86°; the derived anilide had m. p. 133—135°.

Fraction 3 (4·593 g.) was crystalline. It contained 2 : 4 : 6-tri-*O*-methylglucose (97%, by hypoiodite oxidation) {$[\alpha]_D$ +91° ⟶ +72° (*c* 0·92 in H_2O, after 24 hr.); m. p. and mixed m. p. 122—124°}. The corresponding anilide had m. p. and mixed m. p. 160—161°. The fraction was homogeneous on paper chromatography, even after prolonged development. The R_g values (tetra-*O*-methylglucose standard) of authentic 2 : 3 : 4- and 2 : 4 : 6-tri-*O*-methylglucose were 0·85 and 0·80 respectively.

Fraction 4, a syrup (0·491 g.), contained two methylglucoses [paper chromatography, R_g 0·59 (brown spot), R_g 0·62 (pink spot)], and gradually some crystals were formed. The fraction was dissolved in ethyl acetate and, on cooling, 4 : 6-di-*O*-methylglucose (R_g 0·59), m. p. 155—157°, mixed m. p. 154—156° (0·097 g.), was deposited. Evaporation of the ethyl acetate solution yielded a syrup (0·379 g.) which contained a sugar with R_g 0·62.

Since the mixed sugars contained 0·231 g. of tetra-*O*-methylglucose, the proportion of non-reducing end-groups is 4·4% or one per 23 glucose residues.

Degradation of Laminarin by Lime-water.—In extensive small-scale experiments laminarin was treated with lime-water for varying periods, at 25° or 60° in the presence or absence of oxygen. In a large-scale experiment laminarin (12 g.) was treated with saturated oxygen-free lime-water (1500 ml.) at 60° under nitrogen. Samples (5 ml.) were removed at intervals, and the consumption of lime-water determined by titration with 0·01N-hydrochloric acid. Reaction was complete after 23 hr. The mixture was neutralised with dilute nitric acid and concentrated to *ca.* 50 ml. The polysaccharide which was deposited on storage was collected by centrifugation, dissolved in water, and freeze-dried (yield, 5·5 g., 46% recovery). To the remaining solution, ethanol (80% v/v) was added and the resulting precipitate also dissolved in water and freeze-dried (yield, 4·5 g.). This material contained carbohydrates of low molecular weight which gave glucose on acid hydrolysis, saccharinic acid (48%), and non-lactonisable acid (19%) (estimated by the method of Bamford, Bamford, and Collins [29]), and had an ash content of 15%.

The lime-treated laminarin had $[\alpha]_D$ −7° (*c* 5·0 in H_2O), ash, 0·5%, and R.P. 0·3 (Somogyi), 1·7 (potassium ferricyanide); an acid hydrolysate contained mannitol (paper chromatography). Re-treatment with lime-water caused little further degradation, *ca.* 90% of polysaccharide being recovered.

On partial hydrolysis with oxalic acid, the hydrolysate of lime-treated laminarin contained the same series of sugars as that from the original laminarin. In a similar experiment with

[28] Hough, Jones, and Wadman, *J.*, 1949, 2511.
[29] Bamford, Bamford, and Collins, *Proc. Roy. Soc.*, 1950, *A*, **204**, 85.

another sample of lime-treated laminarin, the presence of a reducing sugar with the R_G value of gentiobiose was noted.

Methylation Analysis of Lime-treated Laminarin.—This polysaccharide (8·5 g.) was methylated eight times with dimethyl sulphate and sodium hydroxide, as described for laminarin (p. 3239). The methylated product (10·3 g., 96%) had ash 1·0, OMe, 44·1%. On addition of 94% v/v light petroleum (b. p. 40—60°) to a solution of methylated polysaccharide (9·2 g.) in chloroform (50 ml.), a precipitate (7·5 g., 88%) was obtained, having $[\alpha]_D$ −7·1° (*c* 1·3 in $CHCl_3$). The addition of further light petroleum (1 l.) to the supernatant solution did not give a precipitate.

The original and the precipitated sample of methylated lime-treated laminarin had molecular weights of 2500 and 13,600 respectively.[13]

The sample (4 g.) of high molecular weight was hydrolysed successively with methanolic 4% hydrogen chloride (130 ml.) and N-hydrochloric acid (300 ml.) as described previously, to give 4·4 g. of methylated glucose derivatives. Partition chromatography on a cellulose column gave five fractions:

Fraction 1, a syrup (0·294 g.) contained 2 : 3 : 4 : 6-tetra-*O*-methylglucose and methyl tri-*O*-methylglucoside. The major portion (0·240 g.) was re-hydrolysed with N-sulphuric acid (25 ml.) for 6 hr., neutralised, and concentrated. The resulting syrup was partitioned on a second cellulose column to give two fractions.

Fraction 1a (0·174 g.) contained crystalline 2 : 3 : 4 : 6-tetra-*O*-methylglucose, which after recrystallisation had m. p. 84—86°; the derived aniline derivative had m. p. and mixed m. p. 134—135°. Fraction 1b, a syrup (0·081 g.), contained 2 : 4 : 6-tri-*O*-methylglucose.

Fraction 2 (0·036 g.) contained tetra- (7 mg.) and 2 : 4 : 6-tri-*O*-methylglucose (29 mg.) (paper chromatography).

Fraction 3 (3·676 g.) crystallised. It was chromatographically homogeneous and contained 2 : 4 : 6-tri-*O*-methylglucose (94%, by hypoiodite oxidation). After recrystallisation from ether, the crystals had m. p. 124—126°, mixed m. p. 123—124°, $[\alpha]_D$ +94° ⟶ +73° (*c* 1·68 in H_2O, after 24 hr.). Its aniline derivative had m. p. and mixed m. p. 160—162°.

Fraction 4 (0·375 g.) was partly crystalline, and contained two sugars. One of these, after crystallisation from ethyl acetate, was identified as 4 : 6-di-*O*-methylglucose, m. p. and mixed m. p. 156—159°. The remaining sugar gave a pink colour with aniline oxalate.

Fraction 5 (0·074 g.) was a white amorphous solid which contained mono-*O*-methylglucose and a trace of glucose. It was not further investigated.

The percentage composition of the hydrolysate was: tetra- 5·2, tri- 84·4, 4 : 6-di- 1·5, unidentified di- 7·2, mono-*O*-methylglucose 1·8, indicating the presence of one non-reducing end-group per 20 glucose residues.

Preparation and Properties of Laminaritol.—Potassium borohydride (5 g.) was added to an aqueous solution of laminarin (10 g. in 500 ml.). After 48 hr., the mixture was neutralised (pH 7; acetic acid), and the polysaccharide precipitated with alcohol. The laminaritol was then dissolved in warm water, reprecipitated, redissolved, and finally freeze-dried. The product (9·6 g.) had glucose content 94%, ash content 1·2%, and R.P. 0·1 (Somogyi). A neutralised acid hydrolysate was examined by paper chromatography; comparison with authentic specimens showed the presence of glucose, mannitol, and sorbitol. Laminaritol (5·2 g.) was then treated with saturated lime-water (650 ml.) in an atmosphere of nitrogen at 60° for 23 hr. The solution was cooled, neutralised (pH 7; dilute nitric acid), and concentrated. Polysaccharide was slowly precipitated and after recovery by centrifugation was washed with water and freeze-dried (yield, 4·8 g.) (Found: ash content, 2·7%). Addition of alcohol to the lime-water solution gave a small precipitate (0·58 g.).

Methylation of Laminaritol.—Laminaritol (5 g.) was methylated six times with dimethyl sulphate and sodium hydroxide; the product was isolated by chloroform extraction (yield, 6·2 g.) (Found: OMe, 43·3%). It was dissolved in chloroform (35 ml.), and light petroleum (b. p. 40—60°) added until precipitation ceased (500 ml.). The recovery was 94%. The material (0·4 g.) which was soluble in light petroleum was recovered.

By isothermal distillation,[17] the insoluble material had a molecular weight of 3800.

Periodate Oxidation of Laminarin and Related Compounds.—(a) *Sodium metaperiodate at 2°.* Laminaribiose (150 mg.) and 3-*O*-methylglucose (50 mg.) were oxidised with 0·3M-sodium metaperiodate (10 ml.) in water (final vol. 200 ml.). Formaldehyde could not be detected during 3 days with the chromotropic acid reagent. The production of formaldehyde from

mannitol, under similar conditions, was complete within 1 hr. and the concentration of formaldehyde (1·8 mol.; theory 2·0) remained constant for 44 hr.

Laminarin (90·0 mg.) and laminaritol (74·9 mg.) were oxidised with 0·3M-sodium metaperiodate (2 ml.) in water (23 ml.). The liberation of formaldehyde from laminarin was as follows:

Time of oxidn. (hr.)	1	3	5	12	48
Formaldehyde prodn. (mol.)	0·022	0·023	0·023	0·023	0·025

This is equivalent to an initial release of 0·021 mol.

The results with laminaritol were:

Time of oxidn. (hr.)	22	44	88
Formaldehyde prodn. (mol.)	0·057	0·058	0·060

The production of formic acid from the control experiments was as follows:

Time (hr.)	2	5	24	72	4—6 days	8 days
Formic acid (mol.)						
Laminaribiose	0·50	0·77	1·18	1·51	1·72	2·08
3-*O*-Methylglucose	—	0·28	0·33	0·58	0·68	—

Under these conditions, 1 mol. of formic acid is produced from laminaribiose after *ca.* 14 hr.

The following results were obtained when laminarin (1 g.) was oxidised:

Time (hr.)	5	24	48	96	168
Periodate uptake (mol.)	0·16	0·19	0·19	0·20	0·21
Formic acid (mol.)	0·09	0·10	0·10	0·11	0·11

It follows that 0·10 mol. of acid is released during the initial primary oxidation.

The production of formic acid from laminaritol was 0·12 mol. after 22 and 44 hr., and 0·13 mol. after 66 and 94 hr., representing a total primary oxidation yield of 0·12 mol.

(b) *Sodium metaperiodate at room temperature.* Laminarin and laminaritol (*ca.* 50 mg.), dissolved in water, were treated with 0·3M-sodium metaperiodate (2 ml.) in a total volume of 25 ml. Samples (2 ml.) were analysed at intervals for formaldehyde:

Time of oxidn. (hr.)	6	23	48
Formaldehyde prodn. (mol.)			
Laminarin	0·032	0·040	0·042
Laminaritol	0·064	0·064	0·064

A duplicate analysis gave 0·040 and 0·061 mol. of formaldehyde, respectively, after 16 and 36 hours' oxidation. Lime-treated laminaritol gave 0·061 mol. under similar conditions.

(c) *Sodium metaperiodate at* pH 8 *and room temperature.* The polysaccharides (*ca.* 8 mg.; concentration determined by acid hydrolysis) were dissolved in water, phosphate buffer (0·1M, pH 8: 12·5 ml.), and 0·3M-sodium metaperiodate (2 ml.) in a total volume of 25 ml. The production of formaldehyde was constant after oxidation for 18 hr. and amounted to 0·52 mol. from laminarin, and 0·55 mol. from laminaritol. Under these conditions, the residual polysaccharide was stable, even after oxidation for 9 days. In a further experiment, lime-treated laminaritol gave 0·57 mol. of formaldehyde.

(d) *Potassium metaperiodate at room temperature.* Laminaribiose (35·5 mg.) and mannitol (17·8 mg.) were dissolved in 3% potassium chloride solution (25 ml.), and 0·25M-sodium metaperiodate (25 ml.) was added. The production of formic acid was determined at intervals, and a reagent control was also analysed. Liberation of acid from mannitol ceased after 4 hr. and corresponded to 3·7 mol. Laminaribiose gave the following results:

Time (hr.)	2	4	6	24	48	96	144	168
Formic acid (mol.)	0·38	0·65	0·96	1·38	1·81	2·60	3·08	3·13

Under these conditions (pH 5; cf. Part I), the initial and the total primary oxidation require *ca.* 6 and 130 hr. respectively, and over-oxidation after this time is slight.

Laminarin (235·9 mg.) was oxidised as above:

Time (hr.)	3	6	18	42	168	240
Formic acid (mol.)	0·088	0·096	0·114	0·126	0·156	0·163

The initial and total primary oxidations therefore yield *ca.* 0·10 and *ca.* 0·15 mol. of formic acid, respectively.

Laminaritol (244·9 mg.) was similarly oxidised:

Time (hr.)	2	13	20	38	144	240
Formic acid (mol.)	0·081	0·113	0·122	0·131	0·153	0·168

The total primary oxidation corresponded to *ca.* 0·13 mol., and the rate of production of formic acid was significantly greater than from laminarin. It is apparent that slow overoxidation of laminarin and laminaritol occurs after potassium metaperiodate oxidation for approximately 150 and 100 hr. respectively.

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