162. *The Constitution of Laminarin. Part V.*[1] *The Location of* 1,6-*Glucosidic Linkages.*

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Structural analysis of insoluble laminarin, from *Laminaria hyperborea*, by periodate oxidation and methylation, indicates the presence of 1,6-inter-chain linkages rather than 1,6-inter-residue linkages.

Since a sample of soluble laminarin, from *L. saccharina*, contained more inter-chain linkages than insoluble laminarin, it is suggested that the differences in solubility are due to differences in degree of branching.

The presence of β-1,6-glucosidic linkages in laminarin was first established by Peat and his co-workers [2,3] who isolated small quantities of oligosaccharides containing β-1,6-glucosidic linkages (gentiobiose, 3-*O*-β-gentiobiosylglucose, and 6-*O*-β-laminaribiosylglucose) from a partial acid hydrolysate. The molar ratio of laminaribiose to gentiobiose was 70 to 1, so that the relative proportion of β-1,6-linkages was very low. At first, Peat *et al.*[2] suggested that laminarin, until then believed to possess a linear structure, was in fact branched, and that the β-1,6-linkages were present as branch points. Later, however, they stated [3] that as yet there was no information on the possible branching of the molecule, and suggested a linear structure consisting mainly of β-1,3-linked glucose residues but with occasional β-1,6-inter-residue linkages.

It has been suggested [4] that a proportion of laminarin molecules are branched, since a fraction of methylated laminarin, which had a degree of polymerisation ($\overline{DP}$) of 58 by isothermal distillation,[5] had an average chain-length ($\overline{CL}$) of 23 based on the presence of 4·4% of tetra-*O*-methyl-D-glucose in an acid hydrolysate. Moreover, evidence was obtained [6] for the absence of 1,6-inter-residue linkages since repeated application of the Barry degradation method [7] did not produce small fragments which on dialysis could pass through a cellophane membrane. By contrast, another suggestion [8] was that 1,6-inter-residue linkages were present since ethylene glycol could be isolated after treatment of either the G-chain (glucose terminated) or M-chain (mannitol terminated) fraction of laminarin to periodate oxidation, borohydride reduction followed by a mild acid hydrolysis (to cleave acetal but not glucosidic linkages) and to a second oxidation, reduction, and

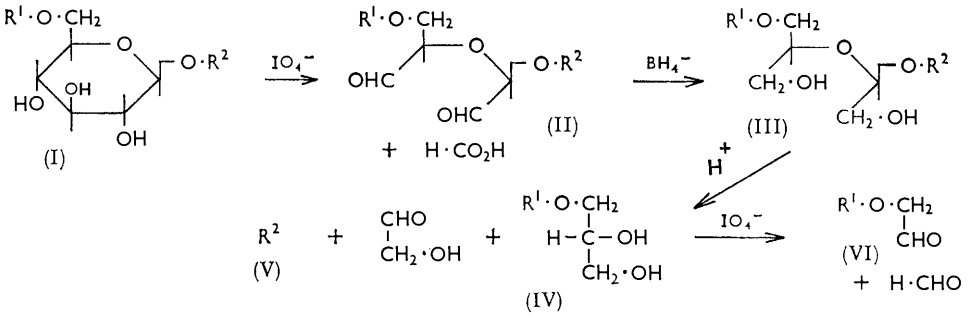
[1] Part IV, Annan, Hirst, and Manners, *J.*, 1965, 220.
[2] Peat, Whelan, and Lawley, *Biochem. J.*, 1953, **54**, xxxiii.
[3] Peat, Whelan, and Lawley, *J.*, 1958, 729.
[4] Anderson, Hirst, Manners, and Ross, *J.*, 1958, 3233.
[5] Broatch and Greenwood, *Chem. and Ind.*, 1956, 1015.
[6] Hirst, O'Donnell, and Percival, *Chem. and Ind.*, 1958, 834.
[7] Barry, *Nature*, 1943, **152**, 537.
[8] Smith and Unrau, *Chem. and Ind.*, 1959, 881.

complete acid hydrolysis. However, we recently showed [1] that ethylene glycol arises from 1-substituted mannitol residues, thus invalidating the above inference with respect to M-chains. In addition, there have been claims [9,10] that small quantities of 2,3,4-tri-*O*-methyl-D-glucose were present in hydrolysates of methylated laminarin, suggesting the presence of 1,6-inter-residue linkages.

The present Paper describes experiments designed to detect the fragmentation of laminarin by the Smith degradation procedure, and a re-examination of methylated laminarin.

The Smith Degradation of Laminarin.—Since the Smith degradation involves periodate oxidation, borohydride reduction, and cleavage of acetal (but not glucosidic) linkages by mild acid hydrolysis, the presence of 1,6-inter-residue linkages will cause fragmentation of the molecule [formulæ (I)—(V)]. Smith and Unrau [8,9] used 0·1N-sulphuric acid at room temperature for 10 hours to hydrolyse acetal linkages. However, recent studies [11] on lichenin polyalcohol suggested that stronger conditions are required for the complete hydrolysis of acetal linkages, and we used a 20 hour hydrolysis.

The polyalcohol from laminarin sample 1 (from *Laminaria hyperborea*) prepared previously [1] was chromatographed on a column of Sephadex G-50, and was eluted as a sharp peak, except for a minor peak amounting to only 3% of the material [Fig. (a)]. After treatment with 0·1N-sulphuric acid for 20 hours, the shape of the major peak was unaltered, and apart from the disappearance of the minor component no difference in heterogeneity could be detected [Fig. (b)]. A control experiment showed that the Sephadex column could separate the acid-treated polyalcohol from the pentasaccharide



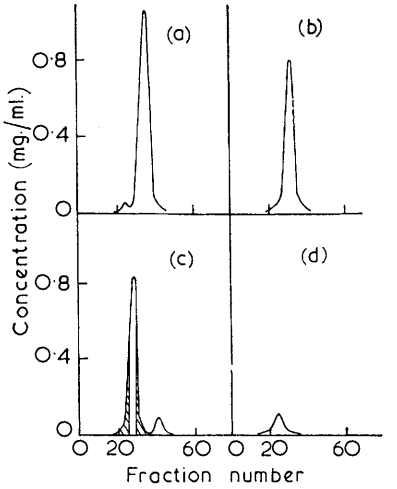
1-*O*-β-laminaritetraosylmannitol [Fig. (c)]. We conclude that no fragmentation of the polyalcohol occurred, although the conditions were suitable for the hydrolysis of acetal linkages since glycerol (arising from non-reducing end-groups) could be detected in later fractions from the column.

The sequence of oxidation, reduction, and mild acid hydrolysis converts reducing glucose end-groups (VII) into 2-substituted D-arabitol residues (IX), in addition to cleaving 1,6-linked glucose residues with the production of 3-substituted D-glycerol residues (IV). Periodate oxidation of a mild acid hydrolysate of laminarin polyalcohol should therefore liberate one molecule of formaldehyde from each original reducing glucose residue (VII), and an additional molecule for every 1,6-inter-residue linkage (I). Since the laminarin sample had a $\overline{DP}$ of 24 and contained 43% of G-chains, the yield of formaldehyde arising from the reducing glucose residue is 0·018 molecule per anhydrohexose residue. [M-Chains, which give rise to ethylene glycol residues, do not yield formaldehyde.]

A partial acid hydrolysate of laminarin polyalcohol was neutralised and freed from glycerol, glycollaldehyde, and sodium sulphate by gel-filtration. On periodate oxidation,

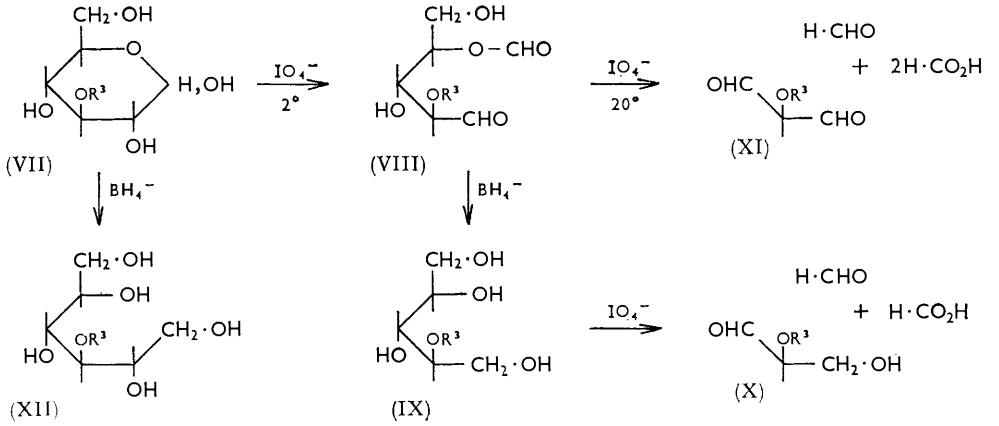
[9] Unrau, Ph.D. Thesis, University of Minnesota, 1959.
[10] Beattie, Hirst, and Percival, *Biochem. J.*, 1961, **79**, 531.
[11] Annan and Manners, unpublished work.

the yield of formaldehyde was 0·019 molecule per anhydrohexose residue. This result provides further evidence for the absence of a significant proportion of 1,6-inter-residue linkages.



Gel-filtration of laminarin polyalcohol on Sephadex G-50. (a) Before, and (b) after mild acid hydrolysis; (c) with reference pentasaccharide; (d) re-chromatography of the leading and trailing edges of the polyalcohol peak from gel-filtration (c). (For details, see Experimental section.)

Examination of Methylated Laminarin.—Assuming complete methylation, the presence of 2,3,4-tri-*O*-methyl-D-glucose in a hydrolysate of methylated laminarin is evidence of 1,6-linked D-glucose residues. In a previous experiment,[4] this sugar could not be detected. A hydrolysate of methylated laminarin has been re-examined by paper and gas chromatography;[12] neither technique gave evidence for the presence of 2,3,4-tri-*O*-methyl-D-glucose. However, when chromatographically pure 2,3,4,6-tetra-*O*-methyl-D-glucose was heated with dilute hydrochloric acid, small amounts of tri-*O*-methyl-D-glucoses and a trace of di-*O*-methyl-D-glucose were produced. It is therefore probable that



R¹, R², R³ = chain of β-1,3-linked D-glucose residues.

hydrolytic demethylation of tetra-*O*-methyl-D-glucose was responsible for the earlier observations.[9,10]

The Degree of Branching in Laminarin.—In view of the above evidence of branching in insoluble laminarin, it was of interest to compare the degree of branching with that in soluble laminarin. The method depends upon a comparison of the $\overline{DP}$ and $\overline{CL}$ of laminarin. In our previous study,[4] the $\overline{DP}$ was measured by periodate oxidation at 20° and determination of the liberated formaldehyde, assuming that the terminal reducing-glucose or

[12] Aspinall, *J.*, 1963, 1676.

mannitol residue gave rise to one molecular proportion of formaldehyde. However, we now find that the liberation of formaldehyde from 3-*O*-substituted glucose residues [as in the G-chains of laminarin, (VII)] is a continuous process, and that a stepwise overoxidation follows the complete oxidation of the reducing glucose residue. On oxidation of laminaribiose and laminaritriose, there is no definite stage when the proportion of reducing glucose residues can be estimated by the release of formaldehyde. Only an approximate indication of the $\overline{DP}$ of laminarin can therefore be obtained by this procedure.

An alternative method involves reduction of laminarin to laminaritol, with conversion of G-chains (VII) into S-chains [sorbitol-terminated chains, (XII)] followed by measurement of the formaldehyde released on periodate oxidation. (M-Chains are unaffected by the reduction, but a knowledge of the proportion of M-chains is required.) In the original description of the method [13] it was stated that the sorbitol residues provided 2 molecules of formaldehyde. However, later workers [14] showed that 3-*O*-methyl-D-glucitol on oxidation at pH 3·6 gave only 1·5 molecules of formaldehyde, owing to the existence of two reaction pathways, instead of the theoretical 2 molecules.

In control experiments, laminaribiitol (0·81 mM) was oxidised with 14·3mM-periodate at 2°, but the immediate release of formaldehyde was only 1·4 molecules. On increasing the periodate concentration four-fold, the formaldehyde release was raised only to 1·6 molecules. The original method [13] cannot therefore be used to measure the $\overline{DP}$. However, by use of one tenth of the periodate concentration (*i.e.*, 1·43mM) and oxidation at room temperature, the yield of formaldehyde from laminaribiitol was reduced to 1 molecule, which is in good agreement with a previous result.[15] Under these conditions, laminaritol would yield one molecule of formaldehyde from both M- and S-chains. Laminaritol was therefore prepared from laminarin samples 1 and 6 (*L. hyperborea* and *L. saccharina*, respectively), and oxidised with periodate at 18°; the initial release of formaldehyde was 0·042 and 0·036 molecule per anhydrohexose residue, equivalent to $\overline{DP}$ values of 24 and 28, respectively. The new figure (24) for the $\overline{DP}$ of *L. hyperborea* laminarin is the same as that obtained [4] by the less rigorous method.

Since laminarin does not contain 1,6-inter-residue linkages, the average chain length ($\overline{CL}$) can be measured from the initial production of formic acid on periodate oxidation, after subtraction of the acid arising from the mannitol residues. Attempts to use a dilute (7·5mM) solution of periodate at 2° for this analysis [16] were not successful, since the rate of the desired oxidation was retarded and other side-reactions were not affected. By use of 75mM-periodate, the initial release of formic acid could be readily measured, and for laminarin samples 1 and 6 this amounted to 0·124 and 0·174 molecule per anhydrohexose unit. On subtraction of the acid arising from the mannitol residues (0·072 and 0·081 mol., respectively, based on mannitol contents of 2·4 and 2·7% [1]), the results corresponded to $\overline{CL}$ values of 19 and 11, respectively. Since the respective $\overline{DP}$ values are 24 and 28, the results indicate the presence on a statistical basis of 1·3 and 2·6 non-reducing end-groups per molecule. The former value is in excellent agreement with the calculated presence of an average of 1·3 non-reducing end-groups per molecule, based on the yield of glycerol produced on acid hydrolysis of the polyalcohol of laminarin sample 1,[1] and the $\overline{CL}$ value of sample 6 has since been confirmed by methylation analysis.[17]

The average number of branch points per molecule for laminarin samples 1 and 6 is therefore 0·3 and 1·6, *i.e.*, in sample 1 most of the molecules are linear and only about 30% contain, on the average, one branch point, whereas in sample 6 all the molecules are branched. Since these samples from *L. hyperborea* and *L. saccharina*, respectively, represent examples of "insoluble" and "soluble" laminarin, it is suggested that

[13] Unrau and Smith, *Chem. and Ind.*, 1957, 330.
[14] Cantley, Hough, and Pittet, *J.*, 1963, 2527.
[15] Clancy and Whelan, *Chem. and Ind.*, 1959, 673.
[16] Cf. Manners and Archibald, *J.*, 1957, 2205.
[17] Fleming and Manners, unpublished work.

differences in solubility of the laminarin samples is due to differences in degree of branching and that in the more highly branched molecules there is a lower degree of linear orientation and intermolecular bonding, with a resultant increase in solubility.

Our conclusion on the location of 1,6-linkages in laminarin is so far confined to our samples of the polysaccharide which were isolated from *Laminaria* sp. Convincing evidence has been provided [18] for the presence of 1,6-inter-residue linkages in laminarin from *Eisenia bicyclis*, including the identification of significant amounts of 2,3,4-tri-*O*-methylglucose on methylation, and the isolation of gentiotriose and gentiotetraose from a partial acid hydrolysate; in addition, this sample of laminarin did not contain mannitol. It therefore seems probable that the term " laminarin " covers a range of glucans, in which the minor structural features differ with the species of marine algæ. It follows that commercial samples of laminarin which may have been isolated from mixed or unidentified species of algæ should not be used for structural studies.[9,19] For example, the tentative identification [19] of the trisaccharide 3,6-di-*O*-β-glucosyl-D-glucose in enzymic hydrolysates of a commercial sample of laminarin, which would suggest the presence of 1,6-inter-chain linkages, requires confirmation, using a laboratory-prepared sample of polysaccharide.

Experimental

Methods and Materials.—The methods used for paper chromatography and periodate oxidation were those described in Part IV.[1] The yields of formaldehyde or formic acid are expressed as mol. per anhydrohexose residue. Laminarin samples **1**, **2**, **3**, **4**, and **6** were those used previously; samples **1** and **6** had been isolated from *L. hyperborea* and *L. saccharina* respectively. Laminaritol and laminaribiitol were prepared by reduction of laminarin and laminaribiose with potassium borohydride, and laminarin polyalcohol was the specimen prepared previously from laminarin sample **1**.

Gel-filtration of Laminarin Polyalcohol.—A column (120 × 1·7 cm.) of Sephadex G-50, which should fractionate polysaccharides in the range $\overline{DP}$ 6—60, was prepared and washed with water for 18 hr. before use. Laminarin polyalcohol (29 mg.) was chromatographed, fractions (5 ml.) being collected, and portions (1 ml.) suitably diluted for analysis by the phenol–sulphuric acid method.[20] The course of elution is shown in Fig. (a), the maximum concentration occurring in fraction **35**. Polyalcohol (*ca.* 50 mg.) in water (14 ml.) was treated with 0·3N-sulphuric acid (6 ml.) at 20° for 20 hr. The solution was neutralised to pH 4 (sodium hyroxide) and a sample (18 mg.) applied to the column. Owing to settling of the column, the rate of flow decreased slowly from 1 ml./min., and in this experiment the maximum concentration of polyalcohol was in fraction **33**. Glycerol was detected by paper chromatography in fractions **50**—**54**.

A mixture of acid-treated polyalcohol (20 mg.) and 1-*O*-β-laminaritetraosylmannitol (2 mg.) was fractionated as before. The resolution of the two carbohydrates is shown in Fig. (c). The residual material present in the leading and trailing edges of the polyalcohol peak (fractions **15**—**26** and **30**—**37**), which together corresponded to about 50% of the polyalcohol, were combined and re-fractionated. A unimodal peak was observed [Fig. (d)] indicating that, although some fractionation had occurred, the proportion of material of $\overline{DP}$ substantially different from the mean value was small.

A portion of mild acid hydrolysate (21·3 mg.) was chromatographed on the Sephadex column, and the polyalcohol free from glycerol, glycollaldehyde, and sodium sulphate (fractions **18**—**40**) collected, concentrated, and freeze-dried. A portion of the polyalcohol (18·4 mg.) was dissolved in water (10 ml.), cooled to 2°, and treated with 0·3M-sodium metaperiodate (0·5 ml.). The yield of formaldehyde was 0·019 mol., compared with a value of 0·018 mol. calculated on the assumption that all the formaldehyde arises from the arabitol residues.

In all the experiments, the recovery of polyalcohol, estimated from the area under the peak, was complete.

Examination of Methylated Laminarin.—Repeated paper chromatography of an acid

[18] Handa and Nisizawa, *Nature*, 1961, **192**, 1078.
[19] Chesters and Bull, *Biochem. J.*, 1963, **86**, 31.
[20] Dubois, Gilles, Hamilton, Rebers, and Smith, *Analyt. Chem.*, 1956, **28**, 350.

hydrolysate of methylated laminarin in ethyl methyl ketone–water–ammonia (*d* 0·880) (200 : 17 : 1, v/v) showed that 2,3,4-tri-*O*-methylglucose ($R_{\text{tetra-}O\text{-methylglucose}}$; R_G 0·72) was absent (cf. R_G 0·59 for 2,4,6-tri-*O*-methylglucose).

Gas chromatography (butanediol succinate polyester) [12] of a methanolysate of methylated laminarin confirmed the absence of 2,3,4-tri-*O*-methylglucose, the relative retention times of which are 2·6 and 3·7.

Chromatographically pure tetra-*O*-methylglucose (8 mg.) was heated at 100° in a sealed tube with N-hydrochloric acid (4 ml.) for 26 hr. Paper chromatography of the cooled, neutralised (silver carbonate) solution showed the presence of small amounts of material with the R_G values of the tri-*O*-methylglucoses, and a trace of di-*O*-methylglucose.

Periodate Oxidation of Laminarin and Related Compounds.—(a) *The production of formaldehyde from laminarin.* Laminarin (*ca.* 35—40 mg.) dissolved in water (10 ml.) was treated with 0·3M-sodium metaperiodate (0·5 ml.) at 18° ± 2°. Samples (1 ml.) were withdrawn at intervals, freed from periodate and polysaccharide by treatment with sodium sulphite and ethanol, and analysed for formaldehyde by the chromotropic acid method.

Time of oxidn. (hr.)		4·5	24	31	53	78
Formaldehyde (mol.)	Laminarin sample 1	—	0·029	—	0·037	—
	,, sample 2	0·027	—	0·032	—	0·034
	,, sample 3	0·031	—	0·039	—	0·043
	,, sample 4	0·034	—	0·039	—	0·045

The oxidation was repeated during 16 days on samples 1 and 6, using 1 ml. of periodate solution:

Time of oxidation (days)		1	4	10	16
Formaldehyde (mol.)	Laminarin sample 1	0·032	0·051	0·076	0·100
	,, sample 6	0·031	0·038	0·056	0·074

Precipitation of sample 1 occurred after 5 days. The results show a continuous formation of formaldehyde from laminarin.

(b) *Laminaribiose and laminaritriose.* Laminaribiose (20·6 mg.) or laminaritriose (4·9 mg.) in water (50 or 10 ml.) was oxidised with periodate solution (final concn. 14·3mM). The reduction of periodate was measured spectrophotometrically [21] on samples (0·5 ml.) diluted to 100 ml.

Time of oxidn. (hr.)		4	21	49	113	169	258
Formaldehyde (mol.)	Laminaribiose	0·14	0·17	0·30	0·54	0·72	0·93
	Laminaritriose	0·13	0·25	0·38	0·66	0·87	—
Redn. of periodate (mol.) by laminaribiose		2·8	3·7	4·2	4·8	5·9	6·8

It is clear that only about 0·40 mol. of formaldehyde was produced after 3 days (cf. ref. 4), and did not approach 1 mol. until after 11 days. A plot of the release of formaldehyde against reduction of periodate by laminaribiose showed the expected initial reduction of 3 mol. of periodate followed by a linear production of formaldehyde.

(c) *Laminaribiitol.* Laminaribiitol (11·6 mg.) in water (20 ml.) was oxidised with periodate (final concn. 14·3mM) at 2°. The disaccharide alcohol (2·9 mg.) was also oxidised with periodate at a final concn. of 57mM.

Time of oxidn. (hr.)		1	4	10	25	47
Formaldehyde (mol.)	with 14·3mM-periodate	1·29	1·36	1·42	1·45	1·46
	with 57mM-periodate	1·57	1·61	1·65	1·70	1·78

By extrapolation, the initial production of formaldehyde was 1·4 and 1·6 mol., respectively.

Laminaribiitol (3·24 mg.) in water (20 ml.) was treated with 0·03M-sodium metaperiodate (1 ml.) at 18°. Samples (1 ml.) were removed for analysis of formaldehyde; samples (2 ml.) were diluted to 50 ml. for measurement for the reduction of periodate.[21]

Time of oxidn. (hr.)	0·5	1·3	2·5	4·5	7
Prodn. of formaldehyde (mol.)	1·00	1·06	1·12	1·17	1·21
Redn. of periodate (mol.)	—	—	2·0	2·2	—

By extrapolation, the initial release of formaldehyde was 1·04 mol. (theory 1·00 mol.).

[21] Aspinall and Ferrier, *Chem. and Ind.*, 1957, 1216.

(d) *Laminaritol.* Dilute solutions of laminaritol from laminarin samples 1 and 6 were oxidised at 18° as follows. Polysaccharide (*ca.* 16 mg.) in water (20 ml.) was treated with 0·03M-sodium metaperiodate (1 ml.) at 18°. Samples (1 ml.) were added to 0·1M-sodium sulphite (0·5 ml.), and ethanol (5 ml.) to remove periodate and polysaccharide. A portion (2 ml.) was analysed for formaldehyde with the chromotropic acid reagent; samples (2 ml.) of the oxidation mixture were diluted to 50 ml. for measurement of the reduction of periodate.

Time of oxidn. (hr.)		0·6	1	2	4	6
Laminaritol 1	Formaldehyde prodn.	0·041	0·043	0·042	0·044	—
	Redn. of periodate	—	—	0·13	—	0·16
Laminaritol 6	Formaldehyde prodn.	0·035	0·036	0·036	0·037	0·041
	Redn. of periodate	—	—	0·13	—	0·17

Since the amount of periodate supplied was 0·32 and 0·28 mol., respectively, an adequate excess of periodate was present. The initial release of formaldehyde was 0·042 and 0·036 mol., respectively, and as control experiments have shown that both M- and G-chains liberate 1 mol. of formaldehyde, the results are equivalent to $\overline{DP}$ values of 24 and 28.

(e) *The production of formic acid from laminarin.* Laminarin samples 1 and 6 (*ca.* 420 mg.) dissolved in water (*ca.* 200 ml.) were oxidised at 2° with 0·1875M-sodium metaperiodate (10 ml.) in a total volume of 250 ml. Samples (25 ml.) were withdrawn at intervals, treated with 12·4% ethylene glycol (5 ml.), and the formic acid content determined by titration with 0·0095N-barium hydroxide to pH 6·3. Samples (2 ml.) were also diluted to 250 ml. for measurement of the reduction of periodate.

Time of oxidn. (hr.)		1	6	19	43	67	100
Laminarin sample 1	Prodn. of formic acid (mol.)	0·07	0·08	0·11	0·12	0·13	—
	Redn. of periodate (mol.)	0·11	0·16	0·21	0·22	0·23	—
Laminarin sample 6	Prodn. of formic acid (mol.)	0·08	0·10	0·14	0·16	0·17	0·17
	Redn. of periodate (mol.)	0·12	0·19	0·26	0·30	0·33	0·34

The amounts of periodate originally present were 0·76 and 0·68 mol., respectively. There was no clear-cut distinction between the rapid initial production of formic acid from triol groups and the subsequent less specific production of acid during non-Malapradian oxidation. However, the release of formic acid after 1 hr. (0·069 and 0·080 mol.) corresponded well with that expected from the oxidation of mannitol residues only (0·072 and 0·081 mol., respectively).

The oxidation was repeated using a ten-fold increase in periodate (*i.e.*, 75mM) in an attempt to increase the rate of the initial Malapradian oxidation. Laminarin (*ca.* 410 mg.) in water (*ca.* 130 ml.) was oxidised at 2° with 0·1875M-sodium metaperiodate (100 ml.) in a final volume of 250 ml. Samples (25 ml.) were withdrawn for analysis.

Time of oxidn. (hr.)		0·5	5	16	25	41
Formic acid (mol.)	Laminarin sample 1	0·092	0·123	0·128	0·132	0·137
	,, sample 6	0·118	0·168	0·175	0·178	0·180

The rate of formic acid production was linear after *ca.* 8 hr. and the initial release corresponded to 0·124 and 0·174 mol., respectively. The proportion of formic acid arising from non-reducing terminal groups was therefore 0·052 and 0·093 mol., equivalent to $\overline{CL}$ values of 19 and 11, respectively.

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