375. *A Comparison of* iso*Lichenin and Lichenin from Iceland Moss* (Cetraria islandica).

By N. B. Chanda, E. L. Hirst, and D. J. Manners.

Purified *iso*lichenin and lichenin, from Iceland moss, have been characterised by methylation and periodate oxidation.

*iso*Lichenin has been found to consist solely of D-glucose residues united by α-1 : 3- and α-1 : 4-glucosidic linkages which are present in the relative proportion of 3 : 2. The molecule appears to be linear, with an average chain length of 42—44 glucose residues. The structure of lichenin as a linear polymer of β-D-glucose containing both 1 : 3- and 1 : 4-linkages in the proportion of 3 : 7 has been confirmed.

Several structural investigations of the polysaccharides of lichens, which are symbiotic organisms in the phylum *Thallophyta*, have been reported recently. The major polysaccharide in Iceland moss (*Cetraria islandica*) is lichenin, which was shown by Meyer and Gürtler [1] to contain D-glucose residues united by 1 : 3- and 1 : 4-linkages. In addition, Iceland moss contains *iso*lichenin and hemicelluloses. The latter are composed of D-galactose, D-mannose, and uronic acid residues.[2] Lichenin and *iso*lichenin, from the Indian lichens *Usnea longissima* and *Roccella montagnei* respectively, are both polymers of D-glucose, although they differ in physical and chemical properties.[3] Pustulan, from *Umbilicaria pustulata*, is a linear glucan in which the component residues are united by β-1 : 6-linkages.[4] In contrast, the alkali-soluble polysaccharides of reindeer moss (*Cladonia alpestris*) are highly branched molecules composed of D-galactose, D-glucose, and D-mannose residues.[5] In this communication, a structural investigation of *iso*lichenin from Iceland moss is described, and previous observations on the structure of lichenin have been confirmed and extended.

iso*Lichenin*.—The presence of *iso*lichenin in Iceland moss has been known for many years. This polysaccharide differs markedly from lichenin in being freely soluble in water, giving a dextrorotatory solution, which is stained blue by iodine. Pringsheim [6] concluded that *iso*lichenin was a glucan related to amylose, but Karrer [7] and Meyer [8] and their co-workers considered it to be a mixture of polysaccharides derived from glucose, galactose, and mannose. Attempts to purify *iso*lichenin were, in general, unsuccessful. In the present study it has been found that *iso*lichenin is, in fact, composed solely of glucose residues.

Iceland moss, freed from fatty materials and lichen acids, was extracted with boiling water. The impure lichenin which came down on cooling was removed and smaller amounts of this polysaccharide were then separated by alternate freezing and thawing

[1] Meyer and Gürtler, *Helv. Chim. Acta*, 1947, **30**, 751.
[2] Buston and Chambers, *Biochem. J.*, 1933, **27**, 1691.
[3] Mittal and Seshadri, *J. Sci. Ind. Res., India*, 1954, **13**, *B*, 244; *ibid.*, 1954, **13**, *A*, 174.
[4] Lindberg and McPherson, *Acta Chem. Scand.*, 1954, **8**, 985.
[5] Aspinall, Hirst, and Warburton, *J.*, 1955, 651.
[6] Pringsheim, *Ber.*, 1924, **57**, 1581.
[7] Karrer and Joos, *Z. physiol. Chem.*, 1924, **141**, 311.
[8] Meyer and Gürtler, *Helv. Chim. Acta*, 1947, **30**, 761.

of the solution. Other contaminating polysaccharides were removed as insoluble alkaline-copper complexes, and further purification was effected by fractional precipitation with acetone of the water-soluble copper complex of *iso*lichenin. After dialysis and treatment with ion-exchange resins, the purified *iso*lichenin was precipitated by alcohol. From 6 kg. of Iceland moss, 2·7 g. of pure *iso*lichenin were finally obtained.

On acid hydrolysis, *iso*lichenin was converted almost quantitatively into glucose. The presence of other sugars and uronic acids could not be demonstrated. In water *iso*-lichenin had an unusually high positive rotation ($[\alpha]_D$ +255°) which may be attributed to α-1 : 3-linked glucopyranose residues (compare nigeran,[9] $[\alpha]_D$ +270° to +283° in N-sodium hydroxide). The *iso*lichenin showed slight reducing action towards alkaline hypoiodite (apparent D.P. 34). The aqueous solution gave a greenish-blue colour with very dilute iodine solution (absorption $\lambda_{max.}$ at 600 mμ), but the " blue value " [10] was too low to permit accurate measurement (B.V. *ca.* 0·01 ; cf. *ca.* 1·30 for amylose and *ca.* 0·10 for amylopectin). The *iso*lichenin reduced 0·4 mole of sodium metaperiodate per glucose residue, indicating that some 60% of the glucose residues were unattacked by periodate. The formic acid produced by potassium metaperiodate oxidation corresponded to 1 mole per 14 glucose residues. If (see below) *iso*lichenin is a linear glucan, and the non-reducing and reducing end-groups give rise to one and two molecules of formic acid respectively, this would be equivalent to an average chain length of 42 glucose residues.

Hydrolysis of the methylated polysaccharide (OMe, 44·4%) gave 2 : 4 : 6- and 2 : 3 : 6-tri-*O*-methyl-D-glucose together with small amounts of 2 : 3 : 4 : 6-tetra- and mixed di-*O*-methyl-D-glucoses. The latter (*ca.* 2%) most probably arose from undermethylation of the polysaccharide (a tri-*O*-methyl glucan has OMe, 45·6%) and from hydrolytic demethylation. The proportion of tetra-*O*-methyl-D-glucose in the products of hydrolysis indicated the presence of 1 non-reducing terminal group per 44 glucose residues. Analysis of the tri-*O*-methylglucose fraction showed that 2 : 4 : 6-tri-*O*-methyl-D-glucose was the major component. From this evidence, and that previously cited, it is concluded that the molecule of *iso*lichenin is unbranched, and contains *ca.* 60% of α-1 : 3- and 40% of α-1 : 4-glucosidic linkages.

Measurements of the viscosity of solutions of *iso*lichenin acetate in chloroform indicated a low molecular weight, the D.P. being of the order of 40—50 glucose residues.

*iso*Lichenin was not attacked by barley or soya-bean β-amylase and it follows that α-1 : 3-glucosidic linkages must be situated near the non-reducing end of the molecule. This evidence makes it clear also that the *iso*lichenin investigated could not be a mixture of an amylose and an α-1 : 3-glucan.

The demonstration of α-1 : 3-glucosidic linkages in *iso*lichenin reveals an unusual structural feature in this glucan, and differentiates it from amylose (compare ref. 6). *iso*-Lichenin thus resembles nigeran, the intracellular polysaccharide synthesised by a strain of *Aspergillus niger*, which contains approximately equal numbers of α-1 : 3- and α-1 : 4-glucosidic linkages.[9] The analogy, however, is not complete in that the average chain length of nigeran is 300—350 glucose residues, and the detailed structures must differ since the proportion of 1 : 3- and 1 : 4-linked residues in *iso*lichenin does not permit an alternating sequence of these linkages such as is present in nigeran.

Lichenin.—Earlier investigations had established a structural similarity between lichenin and cellulose. For example, on acetolysis, cellobiose octa-acetate is formed,[11] and on methanolysis of the methylated polysaccharides, methyl 2 : 3 : 6-tri-*O*-methyl-D-glucoside is obtained,[12] although in both cases the yields from lichenin were smaller than those from cellulose. Nevertheless, lichenin differs from cellulose in being soluble in hot water,[13] and more susceptible to enzymic degradation. In 1947, Meyer and Gürtler [1]

[9] Barker, Bourne, and Stacey, *J.*, 1953, 3084.
[10] Bourne, Haworth, Macey, and Peat, *J.*, 1948, 924.
[11] Karrer, Joos, and Staub, *Helv. Chim. Acta*, 1923, **6**, 800.
[12] Karrer and Nishida, *ibid.*, 1924, **7**, 363.
[13] Haworth, *Chem. and Ind.*, 1939, 925.

provided the first clear evidence of a chemical difference between these polysaccharides. Hydrolysis of methylated lichenin gave tetra-*O*-methyl-D-glucose (0·6%, equivalent to an average chain length of *ca.* 170 glucose residues), and a mixture of 2 : 3 : 6- and 2 : 4 : 6-tri-*O*-methyl-D-glucose, thereby indicating the presence of both 1 : 4- and 1 : 3-linkages. Periodate oxidation showed that *ca.* 27% of 1 : 3-linkages were present. The relative proportions of 1 : 3- and 1 : 4-linkages in lichenin were confirmed by Boissonnas.[14]

In the present investigation two samples of lichenin have been studied. They were composed of D-glucose residues (94—96%) united by β-linkages, as shown by the specific rotation ($[\alpha]_D$ +8° to +10° in N-sodium hydroxide) and by hydrolysis to glucose by exo-β-glucosidase preparations * from *Helix aspersa* and almond emulsin. Partial hydrolysis, with dilute acid or with endo-β-glucosidase preparations from malted barley and *Cladophora rupestris*,[15] gave a series of sugars including glucose, cellobiose, laminaribiose, and higher oligosaccharides (paper chromatography). On periodate oxidation, 0·7 mole of periodate per anhydroglucose unit was consumed.

Chromatographic analysis of a hydrolysate of a methylated lichenin showed the presence of (*a*) tetra-*O*-methyl-D-glucose equivalent to an average chain length of 62 glucose residues, (*b*) 2 : 4 : 6-tri- (*ca.* 30%) and 2 : 3 : 6-tri-*O*-methyl-D-glucose (*ca.* 70%), thus confirming the presence of 1 : 3-linkages, and (*c*) no more than a trace of di-*O*-methyl-D-glucoses, showing the virtual absence of branch points. This sample of lichenin has, therefore, a shorter chain length than that examined by Meyer and Gürtler [1] and this difference probably arises from the more extensive fractionation of the lichen polysaccharides in the present study.

Lichenin does not appear to be a mixture of a β-1 : 3- and a β-1 : 4-glucan. A lichenin acetate was prepared, fractionated, and deacetylated. The regenerated polysaccharide consumed 0·7 mol. of periodate per anhydroglucose residue, indicating that no preferential removal of material containing a larger proportion of 1 : 3-linked glucose residues had occurred. Furthermore, treatment of periodate-oxidised lichenin with *iso*nicotinhydrazide or thiosemicarbazide gave the corresponding polymers,[16] and analysis of these (for N) gave values for the α-glycol content of lichenin similar to those previously obtained. If lichenin was chemically heterogeneous, then a complex of a periodate-oxidised β-1 : 4-glucan would be formed, with a correspondingly higher N content (cf. Barry and his co-workers [16]).

The 1 : 3-linkages appear to be randomly situated in the lichenin molecule. Periodate-oxidised lichenin was reduced to the corresponding polyalcohol with potassium borohydride, and partially hydrolysed with acid. Paper chromatography of the hydrolysate showed the presence of glucose but not of laminaribiose. It is concluded from this evidence that few, if any, sequences of two or more adjacent 1 : 3-linkages were present in the periodate-oxidised lichenin.

The lichenin from Iceland moss is therefore similar to the β-glucans isolated recently from barley [17] and oats,[18] although the relative proportions of 1 : 3- and 1 : 4-linkages and average chain lengths are not identical. Barley β-glucan (D.P. *ca.* 100) contains a higher proportion of 1 : 3-linkages (*ca.* 50%) than oat β-glucan (D.P. *ca.* 180 : 33%) and lichenin.

Experimental

Analytical Methods.—The methods used were those described in earlier papers.[19] The following solvents were used for the paper chromatography of (*a*) unsubstituted sugars, and

* Exo-β-glucosidases cause a stepwise hydrolysis of successive linkages in a β-glucan, in contrast to endo-β-glucosidases, which catalyse random hydrolysis.

[14] Boissonnas, *Helv. Chim. Acta*, 1947, **30**, 1703.
[15] Duncan, Manners, and Ross, *Biochem. J.*, 1956, **63**, 44.
[16] Barry, McCormick, and Mitchell, *J.*, 1954, 3692.
[17] Aspinall and Telfer, *J.*, 1954, 3519.
[18] Acker, Diemair, and Samhammer, *Z. Lebensm.-Untersuch.*, 1955, **100**, 180; **102**, 225.
[19] Manners and Khin Maung, *J.*, 1955, 867; Fleming, Hirst, and Manners, *J.*, 1956, 2831.

(*b*) methylated sugars : butan-1-ol–benzene–pyridine–water [5 : 1 : 3 : 3, v/v; (*a*)], butan-1-ol–ethanol–water [4 : 1 : 5, v/v; (*a*) and (*b*)], and ethyl acetate–pyridine–water [10 : 4 : 3, v/v; (*a*)].

iso*Lichenin*

Isolation of iso*Lichenin*.—Iceland moss (500 g.), after extraction with benzene and methanol (Soxhlet) to remove waxy materials, was treated with cold 2% sodium carbonate solution (1·5 l.) to remove lichen acids. The moss residue was washed free from alkali, and extracted with boiling water (3 l.) for 2—3 hr. After filtering, the solution was allowed to cool slowly. Lichenin was precipitated, and was separated by centrifugation. The solution was concentrated and centrifuged, and residual lichenin removed by repeated freezing and thawing of the solution, the lichenin being partially precipitated during the thawing. Crude *iso*lichenin was isolated by precipitation with acetone, dissolution in cold 1% hydrochloric acid, and reprecipitation with acetone. The product, *iso*lichenin A, had $[\alpha]_D^{17}$ +165° (*c* 0·62 in H_2O) and contained glucose together with small amounts of galactose, mannose, and a pentose. From a total of 4 kg. of moss, 5·2 g. of *iso*lichenin A were obtained. The *iso*lichenin was treated with Fehling's solution, and the copper complex shaken with water (only two-thirds of the complex dissolved). The insoluble material, after regeneration to the polysaccharide with acetic acid, gave, on acid hydrolysis, mannose and galactose and a small amount of glucose. The polysaccharide regenerated from the soluble fraction [Fraction A; yield 2·8 g.] contained glucose and only traces of mannose and galactose. Fraction A had $[\alpha]_D^{17}$ +212° (*c* 0·40 in H_2O). An alkaline copper-complex of Fraction A was then fractionally precipitated with acetone. A gelatinous precipitate was rejected, and a flocculent precipitate collected, dissolved in water, acidified (acetic acid), and reprecipitated with acetone. The product, *iso*lichenin B, on acid hydrolysis gave glucose and no other sugar. An aqueous solution of *iso*lichenin B was dialysed for 2—3 days, passed through basic and acidic ion-exchange resins, and finally precipitated with alcohol (yield 1·9 g.).

In a second series of extractions, 0·8 g. of *iso*lichenin was obtained from 2 kg. of moss.

Examination of iso*Lichenin*.—The *iso*lichenin precipitate was a white fibrous solid. Hydrolysis with 0·5N-hydrochloric acid at 100° for 3—4 hr. gave glucose (96%) and no other sugar (quantitative paper chromatography [20]). No uronic acid could be detected in the polysaccharide (naphtharesorcinol test) or on paper chromatography of the acid hydrolysate. *iso*Lichenin had $[\alpha]_D^{15}$ +255° (*c* 1·0 in H_2O), contained 0·32% of ash and had a low reducing power towards hypoiodite with an apparent D.P. of 34. An aqueous solution was stained greenish-blue with iodine, the absorption spectrum having a maximum at 600 mμ, and a blue-value [10] of 0·01.

Periodate Oxidation of iso*Lichenin*.—The polysaccharide (99·6 mg.) was oxidised with potassium metaperiodate at room temperature, under the conditions described previously.[19,21] The formic acid production was as follows :

Time of oxidation (hr.)	24	48	96	144	168	192
Moles formic acid per $C_6H_{10}O_5$ residue, × 10^2	3·8	4·7	5·7	6·1	6·7	6·9

On the assumption that each molecule of *iso*lichenin gives rise to 3 moles of formic acid, the observed yield of formic acid corresponds to a chain length of about 42 units.

In a second experiment, *iso*lichenin (115·4 mg.) was oxidised with sodium metaperiodate (0·25M; 50 ml.). The periodate uptake was determined, at intervals, by Fleury and Lange's method : [22]

Time of oxidation (hr.)	24	48	72	96
Moles of periodate consumed per anhydroglucose unit	0·40	0·41	0·41	0·42

*iso*Lichenin therefore contains about 60% of periodate-resistant glucose residues.

Acetylation of iso*Lichenin*.—*iso*Lichenin (1·8 g.) was acetylated by Pacsu and Mullen's method [23] giving a chloroform- and acetone-soluble acetate (3·0 g.) [Found : CH_3·CO, 43·8. Calc. for $(C_{12}H_{16}O_8)_n$: CH_3·CO, 44·8%] with $[\alpha]_D^{16}$ +160° (*c* 1·04 in $CHCl_3$) and η_{sp}^{20}/c 0·02254

[20] Flood, Hirst, and Jones, *J.*, 1948, 1679.
[21] Halsall, Hirst, and Jones, *J.*, 1947, 1399.
[22] Fleury and Lange, *J. Pharm. Chim.*, 1933, **17**, 107.
[23] Pacsu and Mullen, *J. Amer. Chem. Soc.*, 1941, **63**, 1487.

(*c* 1·035 in $CHCl_3$) corresponding to an apparent D.P. of *ca.* 43, on the assumption that $Km = 5{\cdot}3 \times 10^{-4}$.[24]

Methylation of iso*Lichenin.*—*iso*Lichenin *O*-acetate (2·7 g.) in acetone solution was methylated four times with dimethyl sulphate and sodium hydroxide solution, and three times with methyl iodide and silver oxide. The partly methylated polysaccharide (OMe, 43·3%) was isolated and then fractionated by the solution method, with successive mixtures of chloroform and light petroleum (b. p. 65—70°), as follows :

Fraction	Petroleum–chloroform	Yield (g.)	OMe (%)
1	95 : 5	—	—
2	90 : 10	0·056	—
3	85 : 15	1·398	43·6
4	80 : 20	0·661	42·2

Fractions 3 and 4 were combined, and methylated by Freudenberg and Boppel's liquid-ammonia method.[25] At the end of the reaction, the neutralised mixture was extracted with chloroform, and the extract filtered, dried, and concentrated. Methylated *iso*lichenin was obtained as a white flocculent precipitate on pouring the concentrated solution into light petroleum (b. p. 40—60°) (Found : Ash, 0·5; OMe, 44·3. Calc. for tri-*O*-methylglucan, OMe, 45·6%). The methylated polysaccharide was then fractionated by the solution method, with chloroform–light petroleum (b. p. 70—80°) as follows :

Fraction	Petroleum–chloroform	Yield (g.)	η_{sp}/c (in $CHCl_3$)	$[\alpha]_D^{14}$ (in $CHCl_3$)	OMe (%)
1	90 : 10	0·036	—	—	—
2	85 : 15	1·42	0·0137	+218°	44·5
3	80 : 20	0·34	0·0184	+216	44·1

Fractions 2 and 3 were combined (Found : OMe, 44·4%).

Hydrolysis of Methylated iso*Lichenin and Separation of Methylated Sugars.*—An acid hydrolysate of a sample (10 mg.) was examined by paper chromatography. 2 : 3 : 4 : 6-Tetra- (R_G 1·00), 2 : 4 : 6-tri- (R_G 0·76), 2 : 3 : 6-tri- (R_G 0·83), and di-*O*-methylglucose were present.

Methylated *iso*lichenin (1·40 g.) was hydrolysed with (1%) methanolic hydrogen chloride (100 ml.) under reflux for 13 hr. (constant rotation). After neutralisation (diazomethane), the hydrolysate was concentrated, and further hydrolysed with boiling 0·5N-hydrochloric acid (100 ml.) for 10 hr. The neutralised and concentrated hydrolysate was extracted with chloroform, and this extract concentrated. The syrup (1·50 g.) was fractionated on a cellulose column[26] (70 × 2 cm.), light petroleum (b. p. 100—120°)–butan-1-ol (6 : 4) saturated with water being used as solvent. Three fractions were isolated, which contained 1·40 g. of sugars (93% recovery). Elution of the column with water gave a fourth fraction (13 mg.) containing traces of mono-*O*-methylglucose and glucose, which was not examined further.

Fraction 1. The syrup (0·161 g.) contained tetra-*O*-methylglucose (18·0%, by hypoiodite oxidation) and methyl glucosides of tri-*O*-methylglucoses. It was rehydrolysed with 0·5N-hydrochloric acid (10 ml.), neutralised, and chromatographed on a second cellulose column, giving fraction 1*a* (19 mg.) and 1*b* (118 mg.). Fraction 1*a* was identified as 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose. After three recrystallisations from light petroleum (b. p. 40—60°), it had m. p. 83—85°, $[\alpha]_D^{17}$ +84° (*c* 0·53 in H_2O). Fraction 1*b* was a mixture of sugars with the same R_G values as 2 : 3 : 6- and 2 : 4 : 6-tri-*O*-methylglucose.

Fraction 2. This fraction (1·205 g.) was a mixture of 2 : 4 : 6- (major component) and 2 : 3 : 6-tri-*O*-methylglucose (paper chromatography). It was 90·3% pure, by hypoiodite oxidation. 2 : 3 : 4- and 3 : 4 : 6-Tri-*O*-methylglucose were absent; the sugars gave a negative Weerman reaction. Part of the fraction (300 mg.) was rechromatographed three times on a cellulose column, giving fraction 2*a* (150 mg.) and 2*b* (50 mg.). Fraction 2*a* was pure 2 : 4 : 6-tri-*O*-methyl-D-glucose. After recrystallisation from ether, it had m. p. 120—122°, $[\alpha]_D^{18}$ +75° (equil.) (*c* 1·1 in H_2O) (Found : OMe, 41·1. Calc. for $C_9H_{18}O_6$: OMe, 41·9%). The corresponding 2 : 4 : 6-tri-*O*-methyl-*N*-phenyl-D-glucosylamine had m. p. 143—145°. Fraction 2*b* was identified as 2 : 3 : 6-tri-*O*-methyl-D-glucose. On recrystallisation from ether, it had m. p. 114—116°, $[\alpha]_D^{17}$ +67° (equil.) (*c* 0·6 in H_2O). The phenylhydrazide of the derived

[24] Staudinger and Reinecke, *Annalen*, 1938, **535**, 95.
[25] Freudenberg and Boppel, *Ber.*, 1938, **71**, 2505.
[26] Hough, Jones, and Wadman, *J.*, 1949, 2511.

2 : 3 : 6-tri-*O*-methyl-D-gluconic acid had m. p. 145° (Found : OMe, 28·9. Calc. for $C_{15}H_{24}O_6N_2$: OMe, 28·4%).

Fraction 3. This material (40 mg.; 70% pure by hypoiodite oxidation) appeared to contain 2 : 3-di-*O*-methylglucose (21%) and other di-*O*-methylglucoses (79%) as indicated by quantitative paper chromatography.

The weights and mol. proportions of sugars were : tetra-*O*-methyl- (0·031 g.; 1 mol.), tri-*O*-methyl- (1·220 g.; 42 mol.) and di-*O*-methyl-glucoses (0·028 g.; ·1 mol.). *iso*Lichenin is therefore unbranched, and contains one non-reducing terminal group per 44 glucose residues.

Action of β-Amylase on iso*Lichenin.*—β-Amylase was prepared from barley by the method of Preece and Shadaksharaswamy.[27] The following digest was prepared : *iso*lichenin (50 mg.), 0·04M-acetate buffer (pH 4·6; 2 ml.), β-amylase solution (0·1%; 0·5 ml.), and water (5 ml.). After 48 hours' incubation at 38°, chromatography failed to show the presence of reducing sugars. The iodine-staining power of the polysaccharide was unchanged. In similar digests containing soluble starch or a mixture of starch and *iso*lichenin, maltose was detected after only 15 minutes' incubation.

A sample of purified soya-bean β-amylase kindly supplied by Professor S. Peat, F.R.S., likewise failed to attack *iso*lichenin.

Lichenin

Isolation of Lichenin.—The material which was precipitated from the hot-water extract of Iceland moss (p. 1954) was freed from *iso*lichenin by repeated precipitations from hot water. Addition of Fehling's solution to an alkaline solution of the lichenin gave an insoluble copper complex; after regeneration (acetic acid), the lichenin was precipitated with acetone and dried (yield 3 g. from 500 g. of moss). The polysaccharide was finally shaken with water for 5—6 hr. and reprecipitated with acetone.

Examination of Lichenin.—The lichenin preparations were white powders, insoluble in cold water, and soluble in hot water, or alkali; they had $[\alpha]_D$ +8° to +10° (*c* 1·0 in N-sodium hydroxide). Paper chromatography of acid hydrolysates gave glucose and no other sugar. Sample I had a glucose content of 94% (by polarimetric determination and quantitative paper chromatography [20]), ash content 0·88%, and a slight reducing action towards alkaline hypoiodite (apparent D.P. 80—86). Sample II had a glucose content of 96% (by cuprimetric titration) and an ash content of 0·23%.

Periodate Oxidation of Lichenin.—(a) *Sodium metaperiodate.* Lichenin (60—70 mg.) was shaken, in the dark, with 0·22M-sodium metaperiodate solution (15 ml.) for periods up to 72 hr. The periodate-uptake was determined as before. After 24, 48, and 72 hours' oxidation, both sample I and II had reduced 0·7 mole of periodate per anhydroglucose residue. Approximately 30% of the glucose residues in lichenin are therefore resistant to periodate oxidation.

(b) *Preparation of* iso*nicotinhydrazide and thiosemicarbazide polymers.* Lichenin (sample II) was treated with sodium metaperiodate for 72 hr. at room temperature, and the periodate-oxidised lichenin then isolated, washed, and dried. 50 Mg. portions, dissolved in hot water (2·5 ml.), were cooled and mixed with *iso*nicotinhydrazide (85 mg.) or thiosemicarbazide (56 mg.) in water (5 ml.). The precipitated polymers were collected, washed and dried : *iso*nicotinhydrazide polymer (Found : N, 11·6, equiv. to 66% of α-glycol groups); thiosemicarbazide polymer (Found : N, 12·7, equiv. to 67% of α-glycol groups).

(c) *Partial hydrolysis of the polyalcohol.* Periodate-oxidised lichenin (Sample II; 40 mg.) was treated with potassium borohydride (25 mg.) in water (2 ml.) for 5 hr. The polyalcohol was precipitated with alcohol, then hydrolysed with 0·5N-sulphuric acid (4 ml.) at 100° for 1·5 hr., and the neutralised concentrated hydrolysate examined by paper chromatography. Glucose was the only sugar present. Under similar conditions, a partial hydrolysate of lichenin contained glucose, laminaribiose, cellobiose and higher oligosaccharides.

Acetylation of Lichenin.—Lichenin (Sample I; 2·5 g.) on acetylation (Pacsu and Mullen's method [23]) gave lichenin acetate (3·4 g.) (Found : ash content 0·5; $CH_3{\cdot}CO$, 44·0. Calc. for $(C_{12}H_{16}O_8)_n$: $CH_3{\cdot}CO$, 44·8%). The acetate (3·0 g.) was fractionated from chloroform solution with light petroleum (b. p. 40—60°), as follows :

Fraction	Yield (g.)	Acetyl (%)	$[\alpha]_D^{16}$ (*c* 1·0 in $CHCl_3$)
1	0·4	43·8	−30°
2	2·2	44·2	−34

[27] Preece and Shadaksharaswamy, *Biochem. J.*, 1949, **44**, 271.

Lichenin acetate (Fraction 2) was deacetylated (sodium methoxide), and the regenerated polysaccharide treated with sodium metaperiodate. 0·7 Mole of periodate per anhydroglucose unit was consumed.

Methylation of Lichenin.—Lichenin (Sample I, 6·0 g.) on methylation (seven treatments with dimethyl sulphate and sodium hydroxide) gave a product with OMe 40·8%. Fractionation, by the solution method, with dry benzene–light petroleum (b. p. 75—80°), gave the following fractions :

Fraction	Petroleum–benzene	Yield (g.)	OMe (%)	$[\alpha]_D^{17}$ (in $CHCl_3$)
1	95 : 5	0·08	41·5	−8·2°
2	90 : 10	2·13	43·9	−8·1
3	85 : 15	1·34	43·1	−7·9
4	80 : 20	0·10	39·8	—

Fractions 2 and 3 were combined, and treated twice with methyl iodide and silver oxide. The product had $[\alpha]_D^{17}$ −8·2° (*c* 0·74 in $CHCl_3$) (Found : ash content 0·81; OMe, 44·4%).

Hydrolysis of Methylated Lichenin and Separation of Methylated Sugars.—Paper chromatography of an acid hydrolysate of 10 mg. methylated lichenin showed the presence of 2 : 3 : 4 : 6-tetra- (R_G 1·00), 2 : 3 : 6-tri- (R_G 0·83), 2 : 4 : 6-tri- (R_G 0·76) and di-*O*-methylglucoses.

Methylated lichenin (3·0 g.) was hydrolysed successively with methanolic and aqueous hydrogen chloride, and the resulting syrup (3·2 g.) fractionated on a cellulose column [26] (90 × 2·5 cm.) as described previously.

Fraction	Yield (g.)	Components	Purity (%, by hypoiodite oxidation)
1	0·455	Tetra-*O*-methylglucose and methyl tri-*O*-methylglucosides	10·3
2	2·469	Tri-*O*-methylglucoses	91·5
3	0·039	Di-*O*-methylglucoses	85·1

A fourth fraction (15 mg.) was eluted from the column with water.

Fraction 1. Rehydrolysis and chromatography gave fraction 1*a* (32 mg.) and 1*b* (410 mg.). Fraction 1*a* crystallised, and was identified as 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose; after recrystallisation from light petroleum (b. p. 40—60°) it had m. p. 83—86°, $[\alpha]_D^{15}$ +83° (*c* 0·67 in H_2O). Fraction 1*b* was a mixture of tri-*O*-methylglucoses.

Fraction 2. Paper chromatography showed that 2 : 3 : 4-tri-*O*-methylglucose (R_G 0·85) was absent. The material gave a negative Weerman reaction, indicating the absence of the 3 : 4 : 6-isomer. Part of fraction 2 (500 mg.) was rechromatographed on cellulose columns; fractions 2*a* (333 mg.), 2*b* (129 mg.), and 2*c* (18 mg.) were obtained. Fraction 2*a* was pure 2 : 3 : 6-tri-*O*-methyl-D-glucose. Recrystallisation from ether gave crystals, m. p. 120—122°, $[\alpha]_D^{16}$ +67° (equil.) (*c* 0·53 on H_2O) (Found : OMe, 40·9. Calc. for $C_9H_{18}O_6$: OMe, 41·9%). The phenylhydrazide of the derived aldonic acid had m. p. 145° (Found : N, 8·3; OMe, 27·7. Calc. for $C_{15}H_{24}O_6N_2$: N, 8·6; OMe, 28·4%). Fraction 2*b* was identified as 2 : 4 : 6-tri-*O*-methyl-D-glucose. After recrystallisation (dry ether), it had m. p. 119—123°, $[\alpha]_D^{17}$ +74° (equil.) (*c* 0·82 in H_2O) (Found : OMe, 40·5. Calc. for $C_9H_{18}O_6$: OMe, 41·9%). Treatment with aniline gave 2 : 4 : 6-tri-*O*-methyl-*N*-phenyl-D-glucosylamine with m. p. 143—144°. Fraction 2*c* was a mixture of tri-*O*-methylglucoses.

The rotation of the tri-*O*-methyl fraction in cold methanolic 2% hydrogen chloride was $[\alpha]_D$ +36° ⟶ −15° constant in 24 hr. (*c* 1·04). An authentic mixture of 2 : 3 : 6- (68%) and 2 : 4 : 6-tri-*O*-methyl-D-glucose (32%) showed $[\alpha]_D$ +57° ⟶ −13° constant in 24 hr. (*c* 1·08 in methanolic 2% hydrogen chloride).

Fraction 3. The syrup contained 2 : 3-di-*O*-methylglucose (53%) together with other di-*O*-methylglucoses (47%) by quantitative paper chromatography. In qualitative experiments, treatment of pure 2 : 4 : 6-tri- and 2 : 3 : 6-tri-*O*-methylglucose with N-hydrochloric acid at 100° for 3 hr. gave about 1% of mixed di-*O*-methylglucoses and a trace of mono-*O*-methylglucose. Fraction 3 is therefore considered to be due to (*a*) hydrolytic demethylation and (*b*) under-methylation.

Since 2·724 g. of methylated sugars contained 0·047 g. of pure tetra-*O*-methylglucose, lichenin has an average chain length of *ca.* 62 glucose residues.

Action of β*-Glucosidase Preparations on Lichenin.*—A fine suspension of lichenin was prepared either by dissolution in 0·2N-sodium hydroxide followed by neutralisation with hydrochloric acid (phenolphthalein) or by warming an aqueous suspension of lichenin to 50°. β-Glucosidase

action was studied qualitatively by incubating a lichenin suspension (1%; 2 ml.), 0·2M-acetate buffer (pH 4·6 or 5·0; 0·5 ml.), and β-glucosidase solution (0·5 or 1·0 ml.) at 37°. Samples were analysed at intervals, by paper chromatography, using authentic specimens of glucose, laminaribiose, and cellobiose as reference compounds.

Type of β-glucosidase	Source of enzyme	Method of prep. ref.	Product(s)
Exo-	Almond emulsin	28	Glucose
Exo-	*Helix aspersa*	29	Glucose
Endo-	Malted barley	30	Glucose, cellobiose, laminaribiose and higher oligosaccharides
Endo-	*Cladophora rupestris*	15	Glucose, cellobiose, laminaribiose and higher oligosaccharides.

In a further experiment, lichenin suspension (3 mg./ml.; 5 ml.), 0·2M-acetate buffer (pH 5·0; 2 ml.), and almond β-glucosidase [28] (10 mg. in water; 1 ml.) were incubated at 35°. After 3 days, 12% conversion into glucose was observed (Shaffer–Somogyi estimation). In a control experiment, laminarin $\{[\alpha]_D +9°$ (*c* 2·7 in N-NaOH)$\}$ gave 15% conversion into glucose.

The authors thank Mr. F. B. Anderson for experimental assistance, and Imperial Chemical Industries Limited and the Distillers Company Limited for grants.

DEPARTMENT OF CHEMISTRY, UNIVERSITY OF EDINBURGH. [*Received, December 27th*, 1956.]

[28] Neuberg and Hassid, *Arch. Biochem. and Biophys.*, 1955, **59**, 405.
[29] Karrer, Staub, Weinhagen, and Joos, *Helv. Chim. Acta*, 1924, **7**, 144.
[30] Euler and Svanberg, *Z. physiol. Chem.*, 1921, **112**, 193; Pringsheim and Leibowitz, *ibid.*, 1923, **131**, 262.