778. *The Structure of Lichenin.*

By Stanley Peat, W. J. Whelan, and J. G. Roberts.

The lichenin extracted by water from Iceland moss has been obtained free from *iso*lichenin and submitted to partial acidic hydrolysis. An examination of the products confirms that the lichenin is an unbranched β-glucan in which 1 : 4- and 1 : 3-links occur together in the same molecule. It is calculated that the ratio of 1 : 4- to 1 : 3-links lies between 2 : 1 and 3 : 1. Evidence is presented which suggests that the lichenin chain is a repeating sequence of β-cellotriose units joined through 1 : 3-bonds. Comment is made on a possible route of the biosynthesis of lichenin.

The β-glucan extractable by cold water from oats has been examined by the same methods and shown to be structurally identical with the lichenin of Iceland moss. The oat lichenin is not, however, associated with an *iso*lichenin.

The purpose of this investigation was to elucidate the constitution of lichenin, a polysaccharide occurring in Iceland moss (*Cetraria islandica*), and to test the validity of the claim [1] that a similar substance exists in oats (*Avena sativa*). Moss lichenin has long been known to be a polyglucose,[2] and the similarity of its structure to that of cellulose was established by methylation analysis [3] and by the isolation of α-cellobiose octa-acetate as a product of acetolysis.[4] The solubility of moss lichenin in water (although only sparing), was recognised as precluding a structure based, like that of cellulose, solely on the β-1 : 4-glucosidic linkage,[5] but apart from an unconfirmed suggestion that 1 : 1- and 4 : 4-linkages were present [6] the problem was not resolved until 1947 when Meyer and Gürtler [7] showed that the hydrolysis products of methylated lichenin included 2 : 4 : 6-tri-*O*-methylglucose as well as the 2 : 3 : 6-isomer recognised previously. There was obtained nearly twice as much of the latter sugar as of the 2 : 4 : 6-isomer.[8] This suggestion that one-third of the linkages were of the 1 : 3-type was confirmed when it was shown that periodate consumption corresponded to the oxidation of only 73% of the glucose units in lichenin,[7] these being the 1 : 4-linked units; the 1 : 3-linked glucose units resist oxidation. Meyer and Gürtler's findings in regard to moss lichenin have since been confirmed and extended by Chanda, Hirst, and Manners,[9] and we are indebted to Professor Hirst for information in advance of publication.

The present investigation had three objectives. First, the determination of the configuration of the 1 : 3-linkages [these had been assumed to be in the β-configuration because of the magnitude of the optical rotation of lichenin (+18°)]. Secondly, a proof that the two linkages, 1 : 4 and 1 : 3, are present in the same molecule. Thirdly, the question of the relative arrangement of the two types of linkages. All three problems are amenable to investigation by the same method, namely, by partial acidic hydrolysis of lichenin followed by fractionation and examination of the oligosaccharides liberated.

The presence of a lichenin-like polysaccharide in oats was reported in 1943 by Morris,[1] who noted a similarity in physical properties between the oat polysaccharide and moss lichenin. In particular, the cupric chloride crystallisation patterns were identical. The structure of this polysaccharide has been investigated in the same way as that adopted for moss lichenin.

Iceland-moss Lichenin.—Lichenin was extracted from tannin-free Iceland moss with

[1] Morris, *J. Biol. Chem.*, 1942, **142**, 881.
[2] Klason, *Ber.*, 1886, **19**, 2541.
[3] Karrer and Nishida, *Helv. Chim. Acta*, 1924, **7**, 363.
[4] Karrer, Joos, and Staube, *ibid.*, 1923, **6**, 800; Karrer and Joos, *Biochem. Z.*, 1923, **136**, 537.
[5] Staudinger and Lantzsch, *J. prakt. Chem.*, 1940, **156**, 65.
[6] Hess and Lauridsen, *Ber.*, 1940, **73**, 115.
[7] Meyer and Gürtler, *Helv. Chim. Acta*, 1947, **30**, 751.
[8] Boissonas, *ibid.*, p. 1703.
[9] Chanda, Hirst, and Manners, *J.*, 1957, 1951.

boiling water and repeatedly recovered from aqueous dispersion by freezing and thawing, to remove, in particular, *iso*lichenin which is stained blue by iodine. Several methods of purification were tested, including precipitation from alkaline solution, treatment with α-amylase, and acetylation. A combination of the last two methods was selected for large-scale working: the polysaccharide was hydrolysed in 0·33N-sulphuric acid to 46% apparent conversion into glucose and the products were fractionated on charcoal–Celite.

The only monosaccharide detected was glucose. The disaccharide fraction was resolved into two components, identified as cellobiose and laminaribiose, in the molar ratio 7·5 : 1 (see Table). The laminaribiose (3-*O*-β-D-glucopyranosyl-D-glucose) in the hydrolysate confirms the presence in lichenin of the 1 : 3-glucosidic linkage and the β-configuration of the link. The ratio of the yield of the two disaccharides cannot be taken as indicating the proportions in which the two types of linkage occur in lichenin since control experiments with laminaritriose and cellotriose showed that β-1 : 3-linkages are more easily hydrolysed by acid than are β-1 : 4-bonds. It should be stressed that the yields of laminaribiose and cellobiose were sufficiently large to preclude the possibility of their being products of acid reversion. The major disaccharide products of glucose reversion are isomaltose and gentiobiose,[10] and the percentage of glucose transformed into disaccharides of all kinds when treated under the same conditions as the moss lichenin was 0·32. The glucose acid reversion products included lævoglucosan (0·42%).[11] The combined yields of laminaribiose and cellobiose represented 18·1% of the original lichenin polysaccharide while lævoglucosan, isomaltose, and gentiobiose were not in fact detected in the lichenin hydrolysate.

The detection of cellobiose and laminaribiose, and these disaccharides only, is strong evidence that the main types of linkage in lichenin are β-1 : 4 and β-1 : 3. The order and arrangement of the two types were investigated by an examination of the tri- and tetrasaccharides liberated by acid hydrolysis. The possible products include five trisaccharides, namely, (*a*) cellotriose, (*b*) laminaritriose, (*c*) 4-*O*-β-laminaribiosylglucose, (*d*) 3-*O*-β-cellobiosylglucose, and (*e*) 3 : 4-di-*O*-β-glucosylglucose. The presence of the sugar (*e*) would be indicative of branching in the lichenin molecule.

The trisaccharide components of the partial acid hydrolysate were eluted from the charcoal column in two fractions, *A* and *B*, with eluant containing 26·5—30% of ethanol. Fraction *A* proved to be pure cellotriose (confirmed by paper chromatrography, specific rotation, and properties of the β-acetate). Fraction *B* was shown to be a mixture containing cellotriose and two other trisaccharides, *B*1 and *B*2, neither of which was laminaritriose. *B*1 and *B*2, having the same R_F value, were not separable by paper chromatography alone, but chromatographically pure *B*1 and *B*2 were obtained by ionophoresis in borate buffer on thick paper. The identical R_F value of *B*1 and *B*2 was consistent with the presence in each of one β-1 : 3- and one β-1 : 4-link, while the ionophoretic M_G values [12] indicate that, of the three trisaccharides (*c*), (*d*), and (*e*), the more mobile *B*1 was probably the trisaccharide (*d*) and *B*2 was the trisaccharide (*c*) or (*e*) or a mixture of both.

The presence in *B*1 and in *B*2 of 1 : 3- and 1 : 4-links was shown by paper-chromatographic separation of their partial acid hydrolysates, zones corresponding to glucose, cellobiose, and laminaribiose being detected in each case (Fig. 1). The trisaccharides *B*1 and *B*2 were actually distinguished by the products of the action of almond emulsin upon them. This β-glucosidase is known to attack oligosaccharides at the non-reducing end linkage.[13] The disaccharides produced by the partial hydrolysis of the trisaccharides (*c*), (*d*), and (*e*) by the glucosidase would therefore be cellobiose from (*c*), laminaribiose from (*d*), and both disaccharides from (*e*) (see Fig. 1). In the actual experiment, trisaccharide *B*1 yielded one disaccharide only which was identified chromatographically

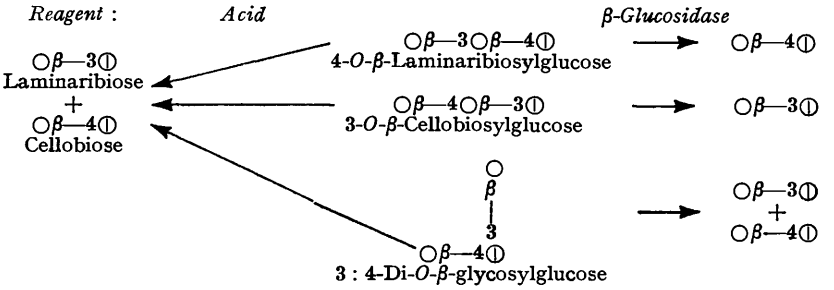
[10] Thompson, Anno, Wolfrom, and Inatome, *J. Amer. Chem. Soc.*, 1954, **76**, 1309.
[11] Peat, Whelan, and Edwards, unpublished work.
[12] Foster, *J.*, 1953, 982.
[13] Edwards, Ph.D. Thesis, University of Wales, 1955.

as laminaribiose, thus confirming the earlier inference that *B*1 was 3-β-cellobiosylglucose (*d*). Trisaccharide *B*2 also gave one disaccharide only and this was identified as cellobiose. It is inferred that *B*2 is 4-β-laminaribiosylglucose (*c*) and that the "branched" trisaccharide (*e*) is not found in the partial acid hydrolysate of lichenin.

The conclusions reached as a result of this study are that moss lichenin is constituted of chains of glucose units linked glycosidically by two types of bond, namely, by β-1 : 4- and β-1 : 3-bonds, that these chains are unbranched, and that the 1 : 3-linkages do not occupy adjacent positions in the chain, since laminaritriose is not produced. The work therefore supports that of Chanda *et al.*[9] who arrived at identical conclusions from a study of the products of hydrolysis of methylated lichenin and from periodate oxidation experiments.

The presence of cellotriose in the partial hydrolysate of lichenin indicates that the distribution of β-1 : 4-links in pairs is not excluded, as appears to be the case with the β-1 : 3-links. A relevant question is as to whether *all* the β-1 : 4-links occur in pairs. Alternative arrangements could be (i) blocks of three or more 1 : 4-links and (ii) single 1 : 4-links flanked by 1 : 3-links. This question would be resolved by a study of the

FIG. 1. *Disaccharide products of the acidic and enzymic hydrolysis of some of the possible structural trisaccharides of lichenin.*



Key : ○ Glucose unit. ① Reducing end glucose unit.

tetrasaccharide components of the hydrolysate since it was to be expected that the tetrasaccharide fraction would include sugar *c*, with the link sequence 1 : 4–1 : 4–1 : 4 (cellotetraose), if arrangement (i) obtained, and sugar *d* with the sequence 1 : 3–1 : 4–1 : 3 in case (ii). It will be appreciated, however, that a rigid analysis of the complex mixture of products of partial acidic hydrolysis and the isolation and identification of the tetrasaccharide components was not practicable. Nevertheless the probable absence of both sugars, *c* and *d*, can be inferred from the following considerations.

From a knowledge of the chromatographic R_F values of the members of the cellodextrin and laminaridextrin series it is possible to calculate the R_F values of sugars containing both 1 : 4- and 1 : 3-linkages (cf. French and Wild [14]). For example, the R_F values found experimentally for the trisaccharides *B*1 and *B*2 agree closely with the calculated values. The R_F value of the tetrasaccharide *d* was calculated in this way and its presence and that of cellotetraose were sought in a lichenin hydrolysate by chromatographic examination.

The search was facilitated by preliminary separation of the components of a partial acid hydrolysate of lichenin into two groups by ionophoresis in borate buffer on thick paper. The slowly moving group, *S*, and the fast moving group, *F*, had M_G values of 0·33 and 0·67 respectively. Paper-chromatographic comparison with cellodextrins and laminaridextrins indicated that the *F* group contained laminaribiose and 3-*O*-β-cellobiosyl-D-glucose (sugar *B*1) as well as glucose (M_G 1·00) while the *S* group contained members having R_F values corresponding to cellobiose, cellotriose, and 4-*O*-β-laminaribiosyl-D-glucose (*B*2). In addition, the chromatogram of *S* showed a tetrasaccharide zone of R_F

[14] French and Wild, *J. Amer. Chem. Soc.*, 1953, **75**, 2612.

value corresponding to that calculated for either the tetrasaccharide with link sequence 1 : 3–1 : 4–1 : 4, or with sequence 1 : 4–1 : 3–1 : 4. It will be observed that the oligosaccharides detected in the ionophoretic group *S* have a terminal 1 : 4-link at the reducing end of the molecule whereas those in group *F* have a terminal 1 : 3-link.[12] If cellotetraose (sugar *c*) or the tetrasaccharide *d* were present in the lichenin hydrolysate it would therefore be expected that the former would be found in the *S* group and the latter in the mobile *F* group. The paper chromatogram afforded no evidence however of the presence of either sugar *c* or *d*.

There is thus some justification for the view that in the lichenin molecule isolated β-1 : 4-linkages or sequences of three or more β-1 : 4-linkages do not occur and that the polysaccharide is constituted of cellotriose units mutually joined by β-1 : 3-links, *i.e.*, a repeating pattern of the link sequence, 1 : 4–1 : 4–1 : 3, as shown in Fig. 2. Such a structure would account for the presence in a partial hydrolysate of the di- and tri-saccharides actually detected, for the absence of laminaritriose and the branched trisaccharide (*e*), and for the probable absence of cellotetraose and the tetrasaccharide *d*.

This conception of the structure of lichenin is also in fair agreement with the proportion of β-1 : 4- to β-1 : 3-linkages determined experimentally. Previous estimates made by periodate oxidation [7,9,15] and by methylation analysis [8,9] all fall within the range 67 to 75%, expressing the proportion of 1 : 4-links as a percentage. Our own estimate, based on periodate oxidation, is 72·5%. Another method of determining the ratio of β-1 : 4- to β-1 : 3-links is provided by comparing the effects of the cuprammonium ion on the specific optical rotations of cellulose and lichenin respectively. If it is assumed [16] that the change of −1060° observed when cellulose is dissolved in cuprammonium solution is due to complex formation with the 2- and 3-hydroxyl groups of the glucose units, then, since the 1 : 3-linked glucose units of lichenin cannot form such complexes, the change in rotation observed with lichenin (−718°) [17] expressed as a percentage of that shown by cellulose is a measure of the proportion of 1 : 4-links: this percentage is 69%.

Oat Lichenin.—The polysaccharide was isolated from oats as described by Morris.[1] By contrast with Iceland moss, the oat lichenin was not contaminated with a soluble iodine-staining fraction corresponding to *iso*lichenin. The structural analysis of the oat polysaccharide was carried out exactly as for moss lichenin, *i.e.*, by partial acidic hydrolysis and fractionation on charcoal–Celite. The mono-, di-, and tri-saccharide components were identical with those obtained from moss lichenin (see Table); 3 : 4-di-*O*-β-glucosylglucose and laminaritriose were absent. A search was also made for cellotetraose and tetrasaccharide *d*, again without success. The oat polysaccharide is clearly similar in structure to, if not identical with, moss lichenin. The only previous information on the structure of oat lichenin was the report,[15] published after this work was complete, that cellobiose octa-acetate is obtained after acetolysis, and the inferential demonstration of the presence of 1 : 3-links in virtue of the incomplete consumption of periodate by the polysaccharide.

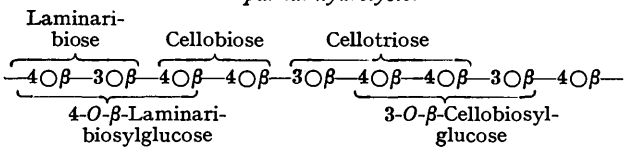
The ratio of 1 : 4- to 1 : 3-links, determined by periodate oxidation, was 76 : 24. This is slightly different from the corresponding ratio for moss lichenin of 72·5 : 27·5. On the other hand Acker, Diemair, and Samhammer [15] found oat lichenin to have a lower proportion (65 : 35) of 1 : 4-linkages than moss lichenin (75 : 25). It may be, however, that the differences simply reflect an inequality in the molecular weights of the two polysaccharides. The ratio of the two linkages is carried out by determining the moles of periodate consumed per glucose residue; a 1 : 4-linked glucose unit consumes one mole of periodate while a 1 : 3 linked unit is unattacked. The end-groups, however, consume more than one mole of periodate and allowance should be made for their presence when making an assessment of the ratio of the two linkages. At the moment we have no information

[15] Acker, Diemair, and Samhammer, *Z. Lebensm.-Untersuch.*, 1955, **100**, 180.
[16] Hess, Waltzien, and Messmer, *Annalen*, 1923, **435**, 1.
[17] Hess and Messmer, *Annalen*, 1927, **455**, 194.

on the molecular weights of our specimens of lichenin. Acker *et al.*[18] state that oat lichenin has a degree of polymerisation of about 380 and that there are 2—3 branch points per molecule. We found no evidence of branching in our structural analysis but would not expect to detect as little as that suggested.

Biosynthesis.—The structure shown in Fig. 2 is a pointer to the possible mechanism of biosynthesis of lichenin, namely, by the polymerisation of β-cellotriose units through 1 : 3-linkages, and may even provide a clue as to the mode of biosynthesis of cellulose. Thus, it may be that the synthesis of cellulose does not follow the usual pattern of polysaccharide biosynthesis, *i.e.*, by the apposition of monosaccharide units to the growing

FIG. 2. *Suggested structure of lichenin, showing the origin of identified oligosaccharide products of partial hydrolysis.*



polymer chain, but may take a form of the type postulated for lichenin, *i.e.*, by polymerisation of cellotriose, or other cellodextrin units, through β-1 : 4-linkages. One case is already on record where a linear glucose polysaccharide containing two different types of linkage has been shown to have these arranged in a regular manner. This is nigeran, in which α-1 : 3- and α-1 : 4-links alternate,[19] and Barker and Carrington [20] have suggested that this might be synthesised *in vivo* from maltose or nigerose or some derivative of these disaccharides. A somewhat similar suggestion has been made to account for the origin of the points of branching in dextran.[21]

Physical properties of the partial hydrolysis products of Iceland moss and oat lichenins.

Sugar	Source *	Yield † (g.)	$[\alpha]_D$ in water	β-Acetate m. p.	β-Acetate $[\alpha]_D$ in $CHCl_3$
Glucose	M	16·3	+52·4°	131—132°	+ 4·0°
	O	2·15	+53·4	131—132	+ 6·0
	A	—	+52·7	135	+ 3·8
Cellobiose	M	7·46	+34·7	194·5—196	− 5·7
	O	1·26	+35·5	192·5—193·5	− 5·9
	A	—	+34·1	193—194	− 7·1
Laminaribiose	M	1·00	+20·3	159—160	−22·6
	O	0·57	+20·1	Syrup	−25·8
	A	—	+18·6	160·5—161·5	−26·1
Cellotriose	M	4·09	+24·3	199·5—200·5	−10·8
	O	‡	+25·0	195·5—196·5	−10·3
	A	—	+23·2	201·5—202·5	−10·2
4-O-β-Laminaribiosylglucose	M	0·74	+12·2	121—123	−22·2
	O	‡	+14·2	—	—
3-O-β-Cellobiosylglucose	M	1·57	+12·7	108—110	−8·3
	O	‡	+11·5	—	—

* M = Moss lichenin, O = oat lichenin, A = authentic specimen.

† From 50 g. of moss lichenin, containing 44·4 g. of carbohydrate, hydrolysed to 46% conversion into glucose, and 25 g. of oat lichenin, containing 22·7 g. of carbohydrate, hydrolysed to 27% conversion into glucose.

‡ Not determined.

EXPERIMENTAL

General Methods

Chromatography and Ionophoresis.—The solvent systems used for descending paper chromatography were butan-1-ol–acetic acid–water (4 : 1 : 5, by vol.), butan-1-ol–pyridine–water

[18] Acker, Diemair, and Samhammer, *Z. Lebensm.-Untersuch.*, 1955, **102**, 225.
[19] Barker, Bourne, and Stacey, *J.*, 1953, 3084.
[20] Barker and Carrington, *J.*, 1953, 3588.
[21] Bailey, Barker, Bourne, and Stacey, *Nature*, 1955, **176**, 1164.

(6 : 4 : 3, by vol.), propan-1-ol–ethyl acetate–water (6 : 1 : 3, by vol.), and butan-1-ol–ethanol–water–ammonia (*d* 0·880) (40 : 10 : 19 : 1, by vol.). It was usual to examine a sugar fraction in two or more of these solvents using Whatman no. 54 paper and to locate the component(s) with benzidine–trichloroacetic acid [22] (reducing sugars) and silver nitrate–sodium hydroxide [23] (reducing and non-reducing sugars). Preparative paper chromatography was carried out on Whatman no. 3 or 3MM paper (18¼ × 22½ in.), up to 100 mg. of sugar being streaked on a 16 in. line parallel to the shorter side of the paper. After irrigation the sugar zones were located by spraying 1 cm. wide strips cut from the sides and middle of the paper. The sugars were eluted from the main body of the paper by pulping the strips with water.

Ionophoresis was carried out in an apparatus of the type described by Latner [24] which would accommodate an 18¼ × 22½ in. sheet of no. 3MM paper. The electrolyte was borate buffer (pH 8·7; 4·45 g. of boric acid and 11·45 g. of borax/l.), and the applied potential of 400 v ensured that a good separation of oligosaccharides having M_G values of 0·32 and 0·67 (see below) was achieved during 18 hr. Preparative electrophoresis was carried out as for paper chromatography except that up to 250 mg. of sugar were accommodated on a single sheet of paper. After ionophoresis the positions of the separated sugars were located as in paper chromatography. Only the benzidine spray could be used; the silver nitrate spray fails to detect sugars on borate-impregnated paper. After elution of the sugar from the dried paper the pH of the extract was adjusted to about 5 with acetic acid to decompose the complex. The salts were then removed by adsorbing the sugar on charcoal–Celite [25] (1 : 1; 50 g. per g. of sugar) and washing with water. The sugar was eluted with 50% ethanol.

Specific Optical Rotations of Sugars and Polysaccharides.—All solutions of sugars were evaporated to dryness under reduced pressure below 50°. The pH was maintained between 5 and 7 by addition of sodium hydroxide or acetic acid. For determination of $[\alpha]_D$ the sugar solutions were treated with Somogyi's deproteinising reagents [26] to remove impurities derived from filter paper or charcoal–Celite,[25] and their concentrations determined by acidic hydrolysis to glucose,[27] which was estimated with Somogyi reagent.[28] Unless otherwise stated, specific optical rotations of sugars and polysaccharides are based on measurements of their glucose contents.

Partial Hydrolysis of Oligosaccharides.—Information about the structures of tri- and tetrasaccharides was obtained by paper chromatography of samples partly hydrolysed by acid and by almond β-glucosidase [29] (emulsin). Acid hydrolysis was carried out by heating the sugar (10 mg.) in 0·33N-sulphuric acid for 1 hr. at 100° and then neutralising the cooled solution with barium hydroxide. For enzymic hydrolysis the sugar (10 mg.) was incubated at 35° with emulsin solution (10 mg. in 1 ml.), and the mixture was then heated to 100° for 5 min. to inactivate the enzyme.

Sugar Acetates.—Acetyl derivatives of sugars were prepared by heating them with sodium acetate–acetic anhydride.

Periodate Oxidation of Lichenin.—Samples of lichenin (about 20 mg. each) were weighed into 10 ml. conical flasks, and 0·2M-sodium metaperiodate (1 ml.) was added. The flasks were shaken in the dark at room temperature (16—21°) at such a rate that the periodate did not come in contact with the rubber stoppers. At periods between 3 hr. and 166 hr. flasks were removed for determination of periodate consumption. The mixture was washed into a 250 ml. flask, 10% potassium iodide (3 ml.) and 2N-sulphuric acid (2 ml.) were added, and the liberated iodine was titrated with 0·0333N-sodium thiosulphate. Two or three determinations were made at each time. When the results were plotted as molecular proportions of periodate consumed per glucose residue against time of oxidation it was found that after the initial rapid oxidation there was a slow linear increase in periodate consumption, presumably owing to over-oxidation. The projection of this line back to the periodate ordinate was used to determine the periodate consumption at the end of the initial oxidation and before the onset of over-oxidation.

[22] Bacon and Edelman, *Biochem. J.*, 1951, **48**, 114.
[23] Trevelyan, Procter, and Harrison, *Nature*, 1950, **166**, 444.
[24] Latner, *Biochem. J.*, 1952, **51**, xii.
[25] Whelan, Bailey, and Roberts, *J.*, 1953, 1293.
[26] Somogyi, *J. Biol. Chem.*, 1945, **160**, 69.
[27] Pirt and Whelan, *J. Sci. Food Agric.*, 1951, **2**, 224.
[28] Somogyi, *J. Biol. Chem.*, 1945, **160**, 61.
[29] Tauber, *ibid.*, 1932, **99**, 259.

Iceland-moss Lichenin

Preparation of Lichenin.—Iceland moss (1 kg.) was broken into small pieces and steeped in 2% potassium carbonate (15 l.) for 24 hr. The brown liquid was then drawn off and the extraction of tannins repeated five times, the final extract being almost colourless. The carbonate was washed away with tap water, and the lichenin then extracted by heating the moss with water (6 l.) in a boiling-water bath for 24 hr. The cooled mixture was filtered through muslin, and the lichenin residue washed with boiling water (3 l.). The lichenin was brought out of solution by alternately freezing (−20°) and thawing the viscous solution several times until the solid became amenable to centrifugation. This material was dissolved in water (4 l.) by autoclaving at 10 lb./sq. in for 1 hr. On cooling, a gel containing suspended polysaccharide was obtained. This was heated to 50° with vigorous stirring and human saliva was added (40 ml.; 20 ml. of saliva diluted with 20 ml. of water and centrifuged). The mixture was cooled to 30° and stored at this temperature for 6 hr.; the pH was 6·5. At the beginning the solution and the suspended solid stained an intense blue with iodine, owing to the presence of *iso*lichenin. After 90 min. the stain had disappeared from the solution but a faint stain persisted in the solid particles until the end of the incubation; then the lichenin was precipitated by freezing to −20°. After thawing, the solid was removed on a muslin filter, and washed successively with water, ethanol, and ether. The air-dried powder (220 g.) contained 30% of moisture and 64·8% of polyglucose. It had $[\alpha]_D^{16}$ +18·4° in 2N-sodium hydroxide (*c* 0·4, based on polyglucose content). The polysaccharide (200 g.) was acetylated as by Morris,[1] being dissolved in boiling water (240 ml.) and precipitated with ethanol (960 ml.) containing 1% of ammonium acetate. The precipitate was washed with ethanol, triturated with pyridine (260 ml.), and centrifuged, and the solid was suspended in pyridine (240 ml.) and acetic anhydride (240 ml.), then heated at 100° for 5 hr. Undissolved material was removed by centrifugation and the supernatant liquid poured into water. The springy product was washed free from pyridine and acetic anhydride, dissolved in acetone, precipitated in water, and dried. The product had $[\alpha]_D^{14}$ −31·7° in $CHCl_3$ (*c* 1·2) [Found: Ac, 42·6. Calc. for $(C_{12}H_{16}O_8)_n$: Ac, 44·8%]. This was de-acetylated by suspension in 2N-sodium hydroxide (1200 ml.) until dissolved (2 hr.), then the brown solution was acidified with acetic acid (1200 ml.) and the lichenin precipitated by pouring into ethanol (12 l.). The precipitate was washed with ethanol and ether and allowed to dry in the air. The pale cream-coloured powder (64 g.) contained 88·8% of polyglucose, 8·8% of moisture, and 1·6% of non-volatile matter. It had $[\alpha]_D^{16}$ +17·7° in 2N-sodium hydroxide (*c* 0·6, based on polyglucose content).

Hydrolysis and Fractionation of Lichenin.—In preliminary experiments the lichenin was dissolved in 2N-sodium hydroxide and then made 0·33N in acid by adding 5N-sulphuric acid. When this solution was heated at 100° hydrolysis was extremely slow, only 7·6% conversion into glucose being achieved in 3 hr. and 12·0% in 10 hr. as compared with 51% conversion of cellotriose and 71% conversion of laminaritriose in 3 hr. in 0·33N-acid. These sugars had not, however, first been dissolved in alkali. The retardation of hydrolysis of lichenin was due to the primary salt effect [30] of the sodium sulphate, present in 0·3N-concentration. It was shown in control experiments that potato starch was 87·6% hydrolysed in 0·33N-acid alone during 2·5 hr. at 100°, whereas when 1·4N-sodium sulphate was also present the conversion fell to 9·4%. Accordingly the lichenin (50 g., containing 44·4 g. of polyglucose) was suspended in 0·33N-sulphuric acid (4 l.) and heated on a boiling-water bath. Samples were removed at intervals for determination of copper-reducing power [28] and heating was stopped after 7 hr.: the apparent conversion into glucose was 45·7%. The cooled, neutralised (sodium hydroxide) solution was evaporated to 500 ml. and adsorbed on a charcoal–Celite column (130 × 5 cm.) which was eluted with water (13·5 l.) from a reservoir 15 ft. above, fractions being collected automatically for 90 min. periods. Initially each fraction was 450 ml. but at the end of the experiment the volume was 150 ml. The elution was continued by the gradient method,[31] the column being fed by a reservoir of water (20 l.) which was continuously replenished through a constant-head device with 40% aqueous ethanol. The optical rotation of each fraction was measured (4 dm. tube), and the fractions were combined as experience suggested, being taken to dryness, dissolved in 80% methanol, filtered, and re-evaporated. After 39 l. of eluate had

[30] Glasstone, " Textbook of Physical Chemistry," 2nd Ed., Macmillan, London, 1948, pp. 1115, 1138.
[31] Alm, *Acta Chem. Scand.*, 1952, **6**, 1186.

been collected the column was eluted with 40% propan-1-ol which removed 9·2 g. of sugar. The total yield of all products was 42·7 g.

Identification of Hydrolysis Products.—(a) *Glucose.* Optically active material first appeared from the column after 2·5 l. of eluate had been collected. The fraction 2·5—7 l. (16·3 g.) behaved chromatographically as glucose, no other sugar being detected, and was identified as such (Table).

(b) *Cellobiose.* The fraction 7—21 l. was free from sugar. Fractions 21—23 l. (2·31 g.) and 23—24 l. (4·37 g.) contained a single disaccharide identified as cellobiose (see Table).

(c) *Laminaribiose.* Fraction 24—26·5 l. (2·29 g.) had $[\alpha]_D^{17}$ +25·9° in H_2O, and paper chromatography showed it to consist of two sugars having the R_F values of cellobiose and laminaribiose respectively. These were separated by ionophoresis on thick filter paper and 0·44 g. of the sugar having M_G value 0·66 was obtained from 0·77 g. of mixture. This sugar was identified as laminaribiose (Table). The relative amounts of cellobiose and laminaribiose in the original fraction were estimated from the value of $[\alpha]_D$, the values of $[\alpha]_D$ +34·8° and 18·6° being assumed for cellobiose and laminaribiose, respectively (see Table).

(d) *Cellotriose.* Fraction 26·5—28·5 l. (0·2 g.) was mainly non-carbohydrate material with a trace of glucose. Fraction 28·5—31 l. (4·09 g.) contained a sugar having the same R_F value as cellotriose. The value of $[\alpha]_D$ was +24·1° on a weight basis and +24·3° based on sugar content as measured by acidic hydrolysis. The crystalline β-acetate was prepared (see Table).

(e) 4-O-β-*Laminaribiosylglucose and* 3-O-β-*cellobiosylglucose.* Paper chromatography of fraction 31—35 l. (2·92 g.) showed it to contain two zones, one with the R_F value of cellotriose and the other moving between cellotriose and laminaritriose. Two zones were also revealed after paper-ionophoretic separation, one moving with cellotriose (M_G 0·32) and one with laminaritriose (M_G 0·67). These two zones were separated by thick-paper ionophoresis. From 1·13 g. of trisaccharide 0·34 g. of the slower-moving component and 0·52 g. of faster-moving component were obtained. The latter substance was chromatographically homogeneous and the means by which it was characterised as 3-*O*-β-cellobiosylglucose are described in the Discussion section.

The component having M_G value 0·32 still behaved chromatographically as did the original mixture and was separated by thick-paper chromatography into cellotriose and the substance of greater R_F value (0·195 g.). This was characterised as 4-*O*-β-laminaribiosylglucose (see Discussion section).

Oat Lichenin

Preparation of Lichenin.—In accord with the procedure of Morris,[1] crude lichenin was extracted from 11 batches of oats (500 g. each). A total of 74·1 g. of material was obtained, the yield in individual extractions varying between 5·40 and 9·70 g. It was confirmed that a glucosan and an araban were present,[2] since glucose and arabinose were released on acidic hydrolysis (evidence from paper chromatography). A sugar with the R_F value of xylose was also detected. The araban was largely removed by taking advantage of its greater solubility in acetic acid, and Morris's method [1] was used to effect this fractionation. In a small-scale experiment 8·7 g. of crude lichenin gave glucan (7·6 g.; moisture content, 28·3%; polysaccharide content, 87·6% of dry wt.; N, 0·67%) and araban (1·1 g.). In a larger-scale experiment crude lichenin (40·7 g.) gave glucan (27·7 g.) and araban (8·86 g.). The final traces of araban were removed from the glucan by a repetition of the acetic acid precipitation. In a third experiment with 25 . of crude lichenin, 14·7 g. of araban-free material were obtained after only one precipitation. The final stage in the purification was the preparation and hydrolysis of triacetyllichenin. Morris [1] acetylated his material with pyridine–acetic anhydride and fractionated the product by precipitation from ethyl acetate. This was repeated on a small scale but it did not seem that any useful purpose was served by this fractionation. The main purpose of the acetate formation was removal of protein. The lichenin (39·4 g.) was acetylated as by Morris [1] and then de-acetylated by suspending it in 2N-sodium hydroxide (600 ml.) until it dissolved (2 hr.); then acetic acid (600 ml.) and ethanol (3 l.) were added. The polysaccharide was centrifuged, washed with ethanol, and dried in a desiccator. The fine white powder (28·7 g.) contained 9·3% of moisture. The dried polysaccharide contained 99·8% of polyglucose, 0·36% of nitrogen, and 0·78% of non-volatile matter. It had $[\alpha]_D^{19}$ +5·6° in 2N-sodium hydroxide (*c* 0·38).

Hydrolysis and Fractionation of Lichenin.—The lichenin (25 g.) was moistened with ethanol (50 ml.) and dissolved in 2N-sodium hydroxide (500 ml.). 5N-Sulphuric acid was added to neutrality, followed by water and a further 170 ml. of acid to a final volume of 2·5 l., so that the acid concentration became 0·33N. The solution was heated on a boiling-water bath and the rate of hydrolysis was followed by cuprimetric estimation of reducing power.[28] After 10 hr., when the apparent conversion into glucose was 27%, the solution was cooled, neutralised with 6N-sodium hydroxide, and concentrated to about 500 ml. The solution was adsorbed on a charcoal—Celite column (128 × 5 cm.) which was eluted with water (10 l.), as for the moss-lichenin hydrolysate, until salts and monosaccharides had been removed. An ethanol gradient was then applied and fractions were collected as for moss lichenin. After fraction 28—33 l. had been collected the column was eluted with 11 l. of 20% propan-1-ol which removed 12·6 g. of oligosaccharides. The total yield of all products was 20·5 g.

Identification of Hydrolysis Products.—(a) *Glucose.* Optically active material appeared after 4·5 l. of water eluate had been collected and persisted until 8 l. The fraction weighed 2·15 g. and had $[\alpha]_D$ +55·8° in H_2O, on a weight basis. When examined by paper chromatography a substance migrating with glucose was detected, together with faint traces of sugars migrating with arabinose and xylose. The last two substances probably arose by contamination with oat araban. Their total amount was estimated at about 10 mg. The main component was then characterised as glucose (see Table).

(b) *Cellobiose.* Fraction 8—18 l. contained neither optically active material nor carbohydrate that could be detected on a paper chromatogram. Fraction 18—20 l. (2·14 g.) was found by paper chromatography to contain cellobiose and laminaribiose. The former was obtained pure by crystallisation from aqueous ethanol (yield, 1·19 g.). The results of characterisation of the cellobiose are shown in the Table. The $[\alpha]_D^{17}$ of the material remaining in the mother-liquors was +23·7° and on the assumption that only cellobiose and laminarobiose were present the relative amounts of each were estimated as 30 : 70 (for method see moss lichenin).

(c) *Laminaribiose.* Fraction 20—21·5 l. (0·71 g.) behaved as a single substance (laminaribiose) on paper chromatography (see Table).

(d) *Cellotriose.* Fraction 21·5—23·5 l. did not contain any carbohydrate. Fractions 23·5—25 l. (0·32 g.) and 25—25·5 l. (0·25 g.) contained only cellotriose (evidence from paper chromatography and ionophoresis). Fraction 25·5—28 l. (1·44 g.) contained cellotriose and a small amount of material of greater R_F value, similar to that encountered in the moss-lichenin trisaccharide fraction. The main component was obtained in a yield of 0·62 g. by crystallisation from aqueous ethanol and identified as cellotriose (see Table). The main component of the mother-liquors was still cellotriose.

(e) *4-O-β-Laminaribiosylglucose and 3-O-β-cellobiosylglucose.* Fraction 28—33 l. weighed 0·95 g. and paper chromatography showed it to consist mainly of a substance migrating between cellotriose and laminaritriose, together with smaller amounts of sugars moving with cello-triose and -tetraose. The material was fractionated by chromatography on thick paper (see moss lichenin) into the main component (0·55 g.), cellotriose (0·16 g.), and the fraction having the R_F value of cellotetraose. Partial acidic hydrolysis showed the last not to be pure cellotetraose since a substance having the same R_F value as the main trisaccharide component was released, in addition to cellotriose. The main component was then separated by ionophoresis on thick paper into components having M_G value 0·32 (0·12 g.) and 0·67 (0·16 g.). These substances were characterised as 4-*O*-β-laminaribiosylglucose and 3-*O*-β-cellobiosylglucose, respectively, by examination of partial hydrolysates produced by acid and by β-glucosidase (see Discussion and Table).

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UNIVERSITY COLLEGE OF NORTH WALES, BANGOR. [*Received, April 29th,* 1957.]