709. *Mannose-containing Polysaccharides. Part V.* The Isolation of Oligosaccharides from Lucerne and Fenugreek Galactomannans.*

By (Miss) Margaret E. Henderson, L. Hough, and T. J. Painter.

Partial acid hydrolysates of the galactomannans of lucerne and fenugreek seeds have afforded in each case 4-*O*-β-D-mannopyranosyl-D-mannose, 6-*O*-α-D-galactopyranosyl-D-mannose, and *O*-β-D-mannopyranosyl-(1 → 4)-*O*-β-D-mannopyranosyl-(1 → 4)-D-mannose. Sucrose and raffinose were found in lucerne seed.

The endosperms of fenugreek and lucerne seeds contain mucilaginous galactomannans which were investigated by Andrews, Hough, and Jones.[1] Results obtained by exhaustive methylation and by periodate oxidation were consistent with a polymer structure involving a chain or backbone of β-1 → 4-D-mannopyranosyl units with D-galactopyranosyl units in attachment at various intervals through their reducing group to $C_{(6)}$ of mannose units. The identification of di- and tri-saccharides produced on partial hydrolysis of these galactomannans is described herein and fully confirms these structural findings.

During the acid hydrolysis of the galactomannans, the liberation was observed first of galactose alone, followed by a mixture of mono- and oligo-saccharides. The oligosaccharides from fenugreek galactomannan were fractionated by adsorption on a column [2] of charcoal–Celite, followed by elution with water, the addition of a displacing agent such as ethanol being unnecessary. Of the oligosaccharides, mannobiose appeared in the effluent first, followed by 6-*O*-α-D-galactopyranosyl-D-mannose and then by mannotriose. The mannobiose and mannotriose were identical with 4-*O*-β-D-mannopyranosyl-D-mannose and *O*-β-D-mannopyranosyl-(1 → 4)-*O*-β-D-mannopyranosyl-(1 → 4)-D-mannose trihydrate isolated by Aspinall, Rashbrook, and Kessler [3] from the mannans of ivory nut.

Examination of the mannobiose by oxidation with sodium metaperiodate under unbuffered conditions and also at pH 3·6 revealed that the reducing unit of the disaccharide reacted in the pyranose form with the formation of a relatively stable formyl ester. The slow breakdown of this ester has been attributed to the presence of the 1 : 4-linkage within the molecule, so preventing the formation of an electrophilic aldehyde group on the carbon atom adjacent to the ester linkage, which, in its presence, is labile.[4]

Partial hydrolysis of lucerne galactomannan gave similar results. Fractionation of the oligosaccharides on an acid-treated, charcoal–Celite column,[5] by gradient elution with water–ethanol, led to the isolation of crystalline 4-*O*-β-D-mannopyranosyl-D-mannose, *O*-β-D-mannopyranosyl-(1 → 4)-β-D-mannopyranosyl-(1 → 4)-D-mannose trihydrate, and 6-*O*-α-D-galactopyranosyl-D-mannose.

Exhaustive extraction of lucerne seed with methanol afforded a mixture of several oligosaccharides which was separated by chromatography on a cellulose column. Sucrose and raffinose [*O*-α-D-galactopyranosyl-(1 → 6)-*O*-α-D-glucopyranosyl(1 → 2)-β-D-fructofuranoside] were characterised, thus suggesting a plausible pathway whereby the galactomannan is synthesised and metabolised as a result of trans-glycosidation of D-galactopyranosyl units, in the first case from raffinose to a mannose polymer and in the second from the galactomannan to sucrose.

Experimental

Paper chromatography was carried out on Whatman No. 1 filter paper by the descending method with the following solvent systems: (*a*) butan-1-ol–ethanol–water (40 : 11 : 19 v/v);

* Part IV, *J.*, 1956, 181.

[1] Andrews, Hough, and Jones, *J.*, 1952, 2744.
[2] Whistler and Durso, *J. Amer. Chem. Soc.*, 1951, **73**, 4189.
[3] Aspinall, Rashbrook, and Kessler, *J.*, 1958, 215.
[4] Hough, Taylor, Thomas, and Woods, *J.*, 1958, 1212.
[5] Alm, Williams, and Tiselius, *Acta Chem. Scand.*, 1952, **6**, 826.

(*b*) ethyl acetate–acetic acid–water (9 : 2 : 2 v/v); (*c*) butan-1-ol–pyridine–water (10 : 3 : 3 v/v); (*d*) propan-1-ol–ethyl acetate–water (7 : 1 : 3 v/v). After separation, reducing sugars were located with *p*-anisidine hydrochloride.[6] Solutions were concentrated under reduced pressure. Optical rotations were observed at 20°, for aqueous solutions.

Selective Hydrolysis of Fenugreek Galactomannan.—The galactomannan (300 g. wet ≡ 68 g. dry) was prepared from milled fenugreek seeds (750 g.) as described previously [1] (Found: sulphated ash, 0·8; N, 0·3%). The polysaccharide was stored in a hydrated condition since it remained more soluble in this form. Preliminary experiments were carried out in order to determine the optimum conditions for di- and tri-saccharide formation. The polysaccharide (≡ 3 g. dry) was suspended in water (120 ml.) by vigorous shaking and heated at 80° ± 1° for 20 hr. in order to ensure complete dispersion. 5N-Hydrochloric acid (30 ml.) was then added with shaking and the heating continued. At hourly intervals two aliquot samples (5 ml. each) were withdrawn simultaneously and examined as follows.

One sample was neutralised with silver carbonate and filtered; hydrogen sulphide was passed through the filtrate, and the solution decolorised with charcoal: any absorbed sugar was eluted from the charcoal with aqueous ethanol. The solution was concentrated and examined on paper chromatograms [solvents (*b*), (*c*), and (*d*)]. During the hydrolysis, galactose appeared first (1 hr.), followed by mannose (2—3 hr.), and then at least six oligosaccharides were clearly distinguishable (3—4 hr.) which gradually disappeared leaving only monosaccharides (6 hr.).

The other sample was neutralised with 0·5N-sodium hydrogen carbonate and to this solution phosphate buffer (20 ml.; pH 11·6 at 25°) and *ca.* 0·1N-iodine (10—30 ml. depending on the reducing power) were added. After sealing with a stopper wetted with potassium iodide solution, the oxidation was allowed to proceed for 48 hr. when after the stopper had been washed the solution was acidified with dilute sulphuric acid and the liberated iodine was titrated with 0·05N-sodium thiosulphate. A control determination was carried out on a sample of the reaction mixture which had not been heated with acid. The results obtained for the increase in reducing power, expressed as aldohexose %, during hydrolysis were 5·85 (1 hr.), 22·9 (2 hr.), 29·6 (3 hr.), 37·3 (4 hr.), and 48·1 (5 hr.).

For the preparation of oligosaccharides, the galactomannan (226 g. ≡ 51 g. dry) was hydrolysed as described above at 80° for 3¼ hr., then the mixture was neutralised with sodium hydrogen carbonate and concentrated to *ca.* 300 ml.; any sodium chloride which had separated was filtered off.

Separation of the Oligosaccharides.—The neutral hydrolysate was passed into a column [2] (120 × 5 cm.) containing a wet mixture of B.D.H. acid-washed charcoal (200 g.) and Celite (200 g.), and elution carried out with water. The effluent from the column was collected in successive portions (*ca.* 100 ml.) which were concentrated and examined on paper chromatograms [solvent (*d*)]; appropriate fractions were combined. Some fractions contained small amounts of charcoal and Celite which were removed either by filtration through Whatman No. 42 paper or on the high-speed centrifuge. Paper chromatography indicated that mannose and galactose were first eluted, followed by two disaccharides (fractions *A* and *B*), a trisaccharide (fraction *C*), and then higher saccharides.

4-O-β-D-*Mannopyranosyl*-D-*mannose.* Fraction *A* was a crisp solid {0·28 g.; $[\alpha]_D$ −9·0° (*c* 1·38)} indistinguishable on paper chromatograms from authentic mannobiose, kindly provided by Dr. G. O. Aspinall. Paper chromatography of a hydrolysate of a small portion showed the presence of mannose only.

Attempts to crystallise the mannobiose from butan-1-ol–ethanol–water failed [cf. Whistler and Durso [2]], but after 8—9 months crystals were obtained which had m. p. and mixed m. p. 192—193°. The phenylosazone was prepared by heating a solution of the mannobiose (50 mg.) in water (2·8 ml.) and acetic acid (0·2 ml.) with phenylhydrazine (0·2 ml.) at 95—100° for 1 hr. After being washed with water and then with benzene, the yellow crystals were dried at 60° under reduced pressure and recrystallised from alcohol–benzene. Calculation [7] of the molecular weight of the phenylosazone from the absorption at 395 mμ gave a value of 515 (calc. for mannobiose phenylosazone: *M*, 520).

Oxidation of mannobiose (0·069 g.) with 0·3M-sodium metaperiodate (10 ml.) in water (made up to 50 ml.) was followed by determining at various intervals of time, the formic acid

[6] Hough, Jones, and Wadman, *J.*, 1950, 1702.
[7] Barry, McCormick, and Mitchell, *J.*, 1955, 222.

liberated [8] and the uptake of oxidant (acid–thiosulphate method [9]). The respective values rose from 2·28 mol. (2 days) to 2·98 mol. (5 days) and from 4·2 mol. (2 days) to 4·75 mol. (5 days). In acetate buffer (pH 3·7) mannobiose rapidly consumed 4 mol. of periodate in 2—3 hr., further oxidation being comparatively slow.

6-O-α-D-*Galactopyranosyl*-D-*mannose*. Fraction *B* was a crisp solid (0·08 g.), a portion of which on hydrolysis with 2N-sulphuric acid for 1 hr. in an autoclave gave galactose and mannose. Crystallisation from butan-1-ol–ethanol–water gave 6-*O*-α-D-galactopyranosyl-D-mannose, m. p. and mixed m. p. 203°, $[\alpha]_D$ +120° (*c* 1·0) (Found: C, 41·5; H, 6·4. Calc. for $C_{12}H_{22}O_{11}$: C, 42·1; H, 6·4%). The disaccharide reacted with 5·97 mol. of sodium metaperiodate during 1 day and yielded 4·98 mol. of formic acid.

O-β-D-*Mannopyranosyl*-(1 → 4)-O-β-D-*mannopyranosyl*-(1 → 4)-D-*mannose trihydrate*. Fraction *C* (0·042 g.) was purified by chromatography on a cellulose column with 50% ethanol as the mobile phase, to give a syrup which slowly crystallised from 80% methanol. After recrystallisation from 50% ethanol, the mannotriose had m. p. 163—165° and mixed m. p. 167°, $[\alpha]_D$ −16° (*c* 0·2; initial value), and gave an *X*-ray powder photograph which was identical with that of an authentic specimen kindly provided by Dr. G. O. Aspinall (Found: H_2O, 9·4. Calc. for $C_{18}H_{32}O_{16},3H_2O$: H_2O, 9·7%). Whistler and Smith [10] report m. p. 137—137·5° and $[\alpha]_D^{25}$ −24·7° → −23·3°, and Aspinall, Rashbrook, and Kessler [3] quote m. p. 134·5—135·5° (rapid heating), 166·5—169·5° (slow heating), $[\alpha]_D$ −15·7° → −20·2°.

Selective Hydrolysis of Lucerne Galactomannan.—The polysaccharide was prepared from powdered lucerne seeds (200 g.) as previously described and dried over phosphoric oxide under reduced pressure (yield, 9 g.). The galactomannan was hygroscopic, absorbing moisture from the atmosphere to the extent of 30% of its own weight. A hydrolysate of a 2% solution of the polysaccharide in 0·1N-hydrochloric acid at 60° was examined at various intervals of time on paper chromatograms. During the first hour, only galactose was detected, after which mannose and oligosaccharides appeared concurrently.

In a typical preparation of oligosaccharides, the galactomannan (5 g.) was hydrolysed at 80° in N-hydrochloric acid (50 ml.) for 3·5 hr. as described above. The mixture was then neutralised by passing through a column of Amberlite resin IR-4B (OH) and evaporated to a syrup.

Separation of the Oligosaccharides.—A solution of the syrup in water (10 ml.) was allowed to soak into a column (50 × 2·8 cm.) containing a wet mixture of B.D.H. decolorising charcoal (40 g.) and Celite (40 g.) which had been treated [5] previously with 6N-hydrochloric acid at 100° for 3 hr. and washed with water until free from acid. Gradient elution with water containing increasing amounts of ethanol was then carried out, and the effluent collected in 5 ml. portions on an automatic fraction-collector; 100 ml. of effluent represented an increment of 2·5% (v/v) in the ethanolic content of the eluate. After examination on paper chromatograms, the appropriate fractions were combined and concentrated in the presence of a little ammonia as the concentrates were apt to become acidic.

4-O-β-D-*Mannopyranosyl*-D-*mannose*. The first fraction {0·09 g.; $[\alpha]_D$ −4·4° (*c*, 1·5); R_{Gal} 0·64 (solvent *b*)} gave only mannose on hydrolysis of a small portion. On oxidation with sodium metaperiodate in the dark, 2·93 mol. of formic acid were released with the consumption of 5·06 mol. of oxidant (constant value) per mol. of mannobiose. In a similar oxidation at pH 3·6 (acetate buffer), 4·07 mol. of periodate reacted. The mannobiose crystallised on concentration of a solution in hot butanol–ethanol–water, and had m. p. and mixed m. p. 192—193°.

6-O-α-D-*Galactopyranosyl*-D-*mannose*. The second fraction {0·11 g.; $[\alpha]_D$ +123° (*c* 1·5); R_{Gal} 0·55 (solvent *b*)} was crystallised by concentration of a solution in methyl Cellosolve–methanol–water and had m. p. and mixed m. p. 202°, $[\alpha]_D$ +122·5 → 122·1 (*c* 1·0; 50 hr.). The derived phenylosazone had m. p. 170°, undepressed on admixture with that prepared from melibiose. Oxidation of the disaccharide with periodate required 40 hr. for complete reaction, when 6·04 mol. of oxidant had reacted and 5·03 mol. of formic acid were liberated.

An unidentified trisaccharide. The third fraction {0·13 g.; $[\alpha]_D$ +32·5° (*c* 3·0); R_{Gal} 0·37 (solvent *b*)} contained galactose (1 mol.) and mannose (2 mol.) as revealed by separation of a hydrolysate of a small portion by paper chromatography followed by determination of the separated monosaccharides by oxidation with alkaline solution of iodine.[1]

O-β-D-*Mannopyranosyl*-(1 → 4)-O-β-D-*mannopyranosyl*-(1 → 4)-D-*mannose trihydrate*.

[8] Halsall, Hirst, and Jones, 1947, 1427.
[9] Hughes and Nevell, *Trans. Faraday Soc.*, 1948, **44**, 941.
[10] Whistler and Smith, *J. Amer. Chem. Soc.*, 1952, **74**, 3795.

The fourth fraction {0·11 g.; $[\alpha]_D$ −22° (*c* 0·72); R_{Gal} 0·31 (solvent *b*)} was indistinguishable from mannotriose on paper chromatograms. Concentration of a solution in propanol–water gave crystals with m. p. and mixed m. p. 167°.

Sucrose and Raffinose from Lucerne Seed.—The milled seed (150 g.) was continuously extracted with methanol (500 ml.) for 36 hr. On evaporation the extract yielded a dark brown syrup which was washed with several portions of benzene, then dissolved in methanol (250 ml.) and decolorised (charcoal). The syrup (12 g.) obtained on evaporation of the solvent contained several oligosaccharides [R_{Gal} 0·88, 0·54, 0·29 (solvent *d*)] as revealed by paper chromatography. Separation of the mixture on a cellulose column,[6] with solvent *a*, gave sucrose (0·65 g.), m. p. and mixed m. p. 180—181°, and raffinose (0·23 g.), m. p. and mixed m. p. 78°, $[\alpha]_D$ +105° (*c*, 1·61).

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