272. *The Galactomannan of the Lucerne Seed.*

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The mucilagenous polysaccharide in the lucerne seed has been shown to consist of a galactomannan containing galactose and mannose in the approximate ratio of 2 to 1. Hydrolysis of the methylated polysaccharide gave a mixture of sugars among which 2 : 3 : 4 : 6-tetramethyl *d*-galactose, 2 : 4 : 6-trimethyl *d*-galactose, and 3 : 4-dimethyl *d*-mannose were detected. The general type of structure present in the polysaccharides is discussed on the basis of this evidence.

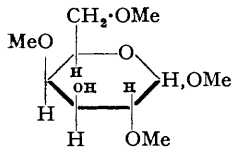
Polysaccharides other than cellulose are of common occurrence in the cell walls of many plants. They are, in general, insoluble in water but form solutions in dilute sodium hydroxide from which they may be precipitated by the addition of alcohol. Investigation of the hemicelluloses from cell walls, with special reference to the endosperm of ungerminated seeds, has revealed the presence of a number of different polysaccharides. Amongst these are the galactomannans which occur in the endosperm of the seeds of *Phœnix dactylifera, Elæis guinensis, Cocos nucifera, Coffea arabia* (Schulze, Steiger, and Maxwell, *Z. physiol. Chem.*, 1890, **14**, 227), and lucerne seed (May and Schulze, *Z. Biol.*, 1936, **97**, 201; Wise and Appling, *Ind. Eng. Chem. Anal.*, 1944, **16**, 28). Galactomannans have also been shown to occur in the seeds of the Fenugreek (Daoud, *Biochem. J.*, 1932, **26**, 255) and in the Carob bean (Spada, *Atte. Soc., Nat. Mat. Modena*, 1939, **70**, 20). It appears that these polysaccharides may function as reserve material in the seed and are utilised by the seed during germination (Schulze, *Landw. Jahrb.*, 1894, **23**, 1; *Ber. deut. bot. Ges.*, 1896, **14**, 66). In view of these observations and since some of the galactomannans have physical properties closely resembling those of the mucilages it was considered of interest to determine the mode of linkage of the sugars in the polysaccharide molecule.

The polysaccharide was isolated from lucerne seeds by the procedure given by Schulze

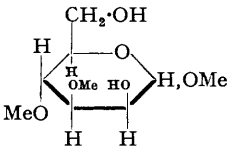
(*loc. cit.*). It was a white powder which did not reduce Fehling's solution and gave aqueous solutions of comparatively high viscosity. It underwent hydrolysis with boiling N-sulphuric acid at a rate indicating the presence of pyranose sugar residues in the molecule, and a quantitative determination of the sugars proved that *d*-mannose and *d*-galactose were present in the approximate ratio of 1 to 2. No other sugar could be detected.

The fully methylated galactomannan was obtained by reaction with sodium hydroxide and methyl sulphate followed by further treatment of the thallium hydroxide complex of the partially methylated derivative with methyl iodide.

The methylated polysaccharide, which was essentially homogeneous, was resistant to methyl-alcoholic hydrogen chloride. Hydrolysis was effected by use of a mixture of hydrochloric acid and glacial acetic acid which has been shown by Bell (*Biochem. J.*, 1935, **29**, 2031) to be efficacious in the hydrolysis of resistant polysaccharides. The products were isolated as the methylglycosides, and on distillation fractions containing the following sugars were obtained: (1) 2 : 3 : 4 : 6-tetramethyl methyl-*d*-galactoside, identified after hydrolysis and formation of the characteristic crystalline anilide of tetramethyl *d*-galactose; (2) 2 : 4 : 6-trimethyl *d*-methylgalactoside (I), identified after hydrolysis as the crystalline sugar and as its crystalline anilide; and (3) 3 : 4-dimethyl *d*-methylmannoside (II), isolated after hydrolysis and oxidation as the crystalline 3 : 4-dimethyl *d*-mannonamide (III). Intermediate fractions were also obtained.



(I.)



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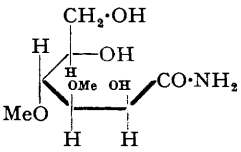

(III.)

The isolation of these sugars proves that the galactomannan is not a mixture of a galactan and a mannan. This follows since a polysaccharide built of solely glycosidically linked mannose residues, some of which are present in a form having three points of linkage with other mannose residues, must possess a corresponding number of residues connected to others only by one linkage. The experimental results show clearly that no such mannose end groups are present, and it follows that the mannose residues must be present in a structure containing galactose also. The sugar residues isolated are all in the pyranose form and are substituted on C_4 by a methoxyl group. The isolation of a large amount of 2 : 3 : 4 : 6-tetramethyl *d*-galactose (*ca.* 30%) proves that the polysaccharide is of the branched-chain type and that half the galactose residues are terminal groupings. Unfortunately, the exact ratios of 2 : 4 : 6-trimethyl *d*-galactose and 3 : 4-dimethyl *d*-mannose could not be accurately determined in these experiments since some of the intermediate fractions which had not been fully examined were lost by enemy action. Nevertheless, an inspection of the methoxyl figures indicates that the relative molecular proportions of 2 : 3 : 4 : 6-tetramethyl methyl-*d*-galactoside, trimethyl methylhexoside, and dimethyl methylhexoside are 1 : 1 : 1. At least one-third of the dimethyl methylhexoside is known to be 3 : 4-dimethyl methylmannoside, and at least one-third of the trimethyl methylhexoside is 2 : 4 : 6-trimethyl methylgalactoside. A further investigation will be necessary in order to determine whether or not other sugars are present amongst the products of hydrolysis. In the meantime it is clear that half the galactose residues are attached in the form Gal . . . and therefore are end groups. Another set of galactose residues is present in the form . . 3Gal . ., linked through positions 1 and 3, and at least one-third of the mannose residues occur as . . $\begin{matrix}6\,M\,1\\2\end{matrix}$ . ., linked through positions 1, 2, and 6.

It will be observed that the 1 : 3 galactose linkage so common in polysaccharides containing galactose, and the 1 : 6 and 1 : 2 mannose linkages occurring in damson gum, cherry gum (Hirst, *J.*, 1942, 70), and yeast mannan (Haworth, Hirst, and Isherwood, *J.*, 1937, 784; Haworth, Hart, and Peat, *J.*, 1941, 833), also occur in this galactomannan.

Experimental.

(All temperatures recorded for distillations are bath temperatures).

Lucerne seeds (500 g.) were finely ground and heated at 100° for 8 hours, with continuous stirring, with 10% potassium hydroxide solution (4 l.) until a jelly-like mass was formed and no more ammonia was evolved. The alkaline solution was then poured with stirring into alcohol (8 l.), and the dark solid which separated was washed with alcohol by decantation and filtered off. The precipitate was

purified by reprecipitation from water with alcohol. Insoluble cell-wall material was then removed by heating the solid with potassium hydroxide solution (4 l.) for 3 hours, cooling, and centrifuging. The insoluble residues were extracted repeatedly until the extracts gave no precipitate on addition of alcohol. The combined extracts were then poured into alcohol (4 vols.) and the precipitate was filtered off and further purified by reprecipitation from acidified aqueous solution (hydrochloric acid) with alcohol.

Traces of starch were removed from the polysaccharide by the action of " taka-diastase " (0·05%) at 37° for 12 hours. Further purification was achieved by precipitation of the copper complex by the addition of copper sulphate solution to a hot alkaline solution of the crude polysaccharide until no further material was precipitated. The copper complex was washed well with hot water and filtered off. The complex was decomposed by grinding it with alcohol containing hydrochloric acid, and the regenerated polysaccharide was ground and washed with alcohol until free from copper and chloride ions. Finally, the product was purified by dissolving it in water and precipitating it from the aqueous solution with alcohol, and dried in vacuum. Yield, 10 g. of a white powder, which dissolved in water to form viscous non-reducing solutions; $[\alpha]_D^{20°}$ + 89° (Found: furfuraldehyde, 2; uronic acid, nil; N, nil; mannose, determined as the phenylhydrazone after hydrolysis with N-sulphuric acid at 95° for 2½ hours, 29·6; galactose, determined as the phenylmethylhydrazone after the same treatment, 58%; equiv. by titration with 0·1N-sodium hydroxide, 4540).

Methylation of the Polysaccharide.—The pure polysaccharide (25 g.) was dissolved in water (300 c.c.) and 30% sodium hydroxide (400 c.c.), and methylated with methyl sulphate (200 c.c.) added in portions (30 c.c.) at 20 minute intervals. The methylation was carried out in an atmosphere of nitrogen and with the usual precautions. After 24 hours the cooled solution was partly neutralised with dilute sulphuric acid and concentrated under reduced pressure. The residue was then re-methylated using the conditions described above. Two repetitions of this process gave a crude methylated product (isolated in the usual manner) which was freed from sodium sulphate and other salts by dialysis. Yield, 15·5 g. (Found: OMe, 34·0%). To complete the methylation the product was dissolved in ethyl alcohol (100 c.c.) and benzene (100 c.c.), and a solution of thallium ethoxide (1·7N, 100 c.c.) in benzene added. Solvent was removed under reduced pressure and the residual solid finely powdered (150 mesh) and boiled under reflux with methyl iodide (150 c.c.) for 30 hours. Excess of methyl iodide was boiled off and the residual solid extracted exhaustively with acetone. Concentration of the extracts gave a residue of partially methylated galactomannan (15 g.) (Found: OMe, 36·2%). This methylation process with thallium ethoxide and methyl iodide was repeated (see above), and the isolated product (13 g.) methylated with silver oxide (10 g.) and methyl iodide (30 c.c.). Repetition of this process gave a product (13 g.) (Found: OMe, 43·5%) isolated in the usual manner.

Fractionation of the Methylated Polysaccharide.—The methylated polysaccharide (10·1 g.) was fractionally precipitated from chloroform by the addition of light petroleum (b. p. 40—60°) giving: Fraction I (2·1 g.), a white solid, $[\alpha]_D^{20°}$ + 72° (*c*, 1·0 in chloroform) (Found: OMe, 43·4%; furfuraldehyde, nil); Fraction II (6·0 g.), a white solid, $[\alpha]_D^{20°}$ + 71° (*c*, 1·1 in chloroform) (Found: OMe, 43·2%; furfuraldehyde, nil); Fraction III (0·9 g.), a white solid, $[\alpha]_D^{20°}$ + 69° (*c*, 0·9 in chloroform) (Found: OMe, 44·1%; furfuraldehyde, nil); Fraction IV (1·0 g.), a sticky solid.

Hydrolysis of the Methylated Polysaccharide.—Hydrolysis of the methylated material was difficult as the product was relatively stable to boiling methyl-alcoholic hydrogen chloride and to hot N-hydrochloric acid. The following procedure was eventually adopted as the most satisfactory technique. The methylated polysaccharide (7·3 g., Fractions I and II) was dissolved in a mixture of glacial acetic acid (50 c.c.), water (25 c.c.), and concentrated hydrochloric acid (25 c.c.), and heated at 100° for 6 hours. Changes in optical rotation were not observable, but preliminary experiments had shown that the hydrolysis was complete at the end of this time. Barium carbonate was added to the cooled solution until a test portion of the solution gave a grey and not a blue colour to Congo-red paper. The solution was then filtered and evaporated to dryness under reduced pressure. The solid residue was then extracted with chloroform, and the extracts were concentrated and boiled with methyl-alcoholic hydrogen chloride (3% w/v) for 12 hours. The cooled solution was then neutralised with silver carbonate and filtered, and the filtrate was concentrated under reduced pressure at 40° to a syrup (6·4 g.) which was fractionally distilled in a vacuum giving: Fraction i (0·86 g.), b. p. 90°/0·001 mm., $n_D^{10°}$ 1·4539 (Found: OMe, 59·5%); Fraction ii (0·72 g.), b. p. 108°/0·001 mm., $n_D^{10°}$ 1·4522 (Found: OMe, 61·9%); Fraction iii (0·53 g.), b. p. 110°/0·001 mm., $n_D^{9°}$ 1·4542 (Found: OMe, 60·0%); Fraction iv (0·57 g.) b. p. 120°/0·01 mm., $n_D^{9°}$ 1·4560 (Found: OMe, 53·8%); Fraction v (1·40 g.), b. p. 130°/0·001 mm., $n_D^{9°}$ 1·4601 (Found: OMe, 48·8%); Fraction vi (1·32 g.), b. p. 160/0·001 mm., $n_D^{9°}$ 1·4680 (Found: OMe, 41·8%); Fraction vii (0·57 g.), b. p. 170°/0·01 mm., $n_D^{9°}$ 1·4780 (Found: OMe, 41·9%); residue, 0·43 g.

Hydrolysis of the Fractions.—The first three fractions having very similar methoxyl values were combined (2·1 g.) and hydrolysed by heating with 2N-hydrochloric acid at 95° for 3 hours; $[\alpha]_D^{20°}$ (initial value not observable), + 71° (2 hours); + 105° (3 hours, constant value). The solution was cooled, neutralised with barium carbonate, and filtered, the filtrate concentrated under reduced pressure, and the residue exhaustively extracted with chloroform. Concentration of the extracts gave a syrup (1·9 g.) which was at least 94% 2 : 3 : 4 : 6-tetramethyl *d*-galactose; $[\alpha]_D^{20°}$ + 109° (in water) (Found: OMe, 52·2. Calc. for $C_{10}H_{20}O_6$: OMe, 51·8%). The sugar (0·32 g.), on being heated under reflux with aniline (0·1 c.c.) dissolved in alcohol (3 c.c.) for 2½ hours, gave 2 : 3 : 4 : 6-tetramethyl *d*-galactose anilide (0·34 g.), m. p. 189°, not depressed on admixture with an authentic sample. Fraction iv (0·56 g.) was hydrolysed by boiling with 2N-hydrochloric acid (25 c.c.) for 3 hours; $[\alpha]_D^{20°}$ + 56° (2 hours); + 70° (3 hours, constant value). The sugar (0·5 g.) was isolated as described above; $[\alpha]_D^{20°}$ + 70° (*c*, 1·0 in water) (Found: OMe, 40·0. Calc. for $C_9H_{18}O_6$: OMe, 41·8%). On standing, the syrup partly crystallised. The crystals were separated by trituration with ether and had m. p. 105°, not depressed on admixture with an authentic specimen of 2 : 4 : 6-trimethyl *d*-galactose. The syrup (0·4 g.) on boiling with aniline (0·3 g.) in ethyl alcohol (2 c.c.) for 2½ hours gave the anilide of 2 : 4 : 6-trimethyl *d*-galactose (0·5 g.), m. p. 179°, not depressed on admixture with an authentic specimen. At the most only traces of 2 : 3 : 6-trimethyl *d*-galactose could be present in the syrup, as on standing with cold 5% methyl-alcoholic hydrogen chloride no downward change of rotation was observable; $[\alpha]_D^{20°}$ + 41° (initial

value); + 49° (3 hours); + 58° (6 hours). Fraction vii (0·57 g.) was hydrolysed with boiling 2N-hydrochloric acid; $[\alpha]_D^{20°}$ + 31° (initial value); 0° (3 hours, constant value). The solution was cooled and neutralised with barium carbonate, and the filtrate was evaporated to dryness. The syrup (0·5 g.), isolated in the usual manner, did not crystallise. The absence of any derivative of mannose or galactose with a hydroxyl group on C_4 was indicated by the fact that the syrup (0·03 g.) in methyl-alcoholic hydrogen chloride (5 c.c.; 5%) showed no change of rotation (+ 10°) in 7 days. The syrup (0·2 g.) was oxidised with bromine water at 45° until non-reducing (7 hours). The cooled solution was neutralised with silver carbonate and filtered, and silver ions were removed with hydrogen sulphide. The filtered solution was concentrated to a syrup (0·15 g.) which did not crystallise. Accordingly, the material was dissolved in liquid ammonia and excess of ammonia allowed to evaporate. The residue crystallised, and by trituration with acetone 3 : 4-dimethyl *d*-mannonamide, m. p. 139°, not depressed on admixture with an authentic specimen, was isolated. The amide gave a strong positive test for an α-hydroxy-amide by the method of Weerman.

A complete examination of Fractions v and vi was not possible since these fractions were lost by enemy action. They obviously contained some 2 : 4 : 6-trimethyl *d*-galactose and 3 : 4-dimethyl *d*-mannose, but at this stage the presence of other galactose and mannose derivatives cannot be excluded.

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