THE MOLECULAR STRUCTURE
OF CARBOHYDRATES IN
SOYBEANS.

by

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TO MY PARENTS

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CONTENTS

SECTION II: Polysaccharides isolated from Soybean Cotyledon Meal.

Experimental

GENERAL INTRODUCTION

Introduction

The soybean (Glycine max), first referred to in the Chinese materia medica written over 4,500 years ago, is a staple food of the Orient where it has been cultivated for thousands of years.

The seed was introduced into the United States of America at the beginning of the 20th century, and from that time the rapid expansion in its cultivation has made America the world's leading producer and exporter of this important oilseed crop.

The readily extractable oil is used in the food, paint and printing ink industries. The meal which is primarily used as a cattle feed also makes a high-protein flour, a sauce, a vegetable milk and a type of cheese; more modern applications are adhesives, plastics and textile fibre.

Extensive studies have been made on the oil (1), protein (2), and amino acid (3) content of the soybean but little is known of the detailed chemistry of the carbohydrate components.

According to Kawamura, defatted and dehulled soybeans contain 7.46% sugars (4) consisting of sucrose, raffinose and stachyose (5). Verbascose, a galactosylstachyose, has been reported (6,7) as a component part of the ungerminated seed.

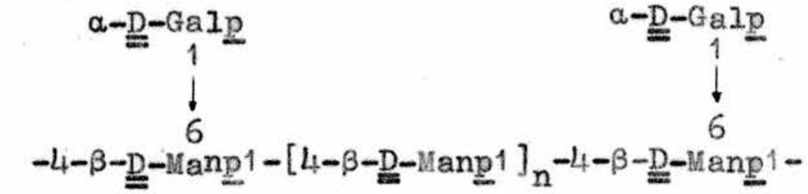
Studies on the evolution of galactosides during formation and germination of the seeds have shown (7) that the total galactosides consisting of raffinose, stachyose and verbascose are the last carbohydrates to be formed in the seed and the first to be utilised as nutrient to the young seedlings. Of the constituent units from the oligosaccharides, D-fructose and D-glucose were readily detectable in extracts from the germinating bean but only traces of galactose could be found. This observation was interpreted to indicate a rapid utilisation of the galactose moiety from the oligosaccharides during the process of germination (8). Chromatographic examination of the glycosidic extracts of the petioles, stems and roots of the bean indicated (9) that maltose was an important glycosidic constituent of the growing plant.

MacMasters (10) determined the percentage of sugars present in a variety of soybeans and found that plants of the same variety grown under different conditions often show distinct variations in chemical constituents; also the plants of the same species but of different varieties exhibit even greater variation. The total sugar content of the bean was shown to decrease with increasing maturity, from a value of about 20% sugar content in the green bean to under 10% in the mature bean. Several polysaccharide fractions were isolated, all of which gave on hydrolysis galactose, arabinose and galacturonic acid. The polysaccharides of the soybean were therefore considered to be a mixture of galacto-arabans containing galacturonic acid.

Starch has been reported in the mature bean in amounts varying from none to 6% (10), but it appears that formation of this polysaccharide increases rapidly during the process of germination (11). The effect of temperature, during germination, on the crystalline structure of starch granules in soybean seedlings has been studied (12). The results showed that the crystalline type of the starch obtained from the cotyledons and hypocotyls varied greatly with the temperature at which germination took place. A transition series from the <u>B</u>-type (found mainly in tuber starches) to one very similar to the <u>A</u>-type (found mainly in cereal starches) was obtained with elevation of the germination temperature. No difference could be observed in the crystalline type of the starch in the cotyledons and hypocotyls of the same seedlings.

Kawamura has reported (13) that treatment of the soybean meal with 0.2% sodium hydroxide effectively solubilises most of the protein and all of the sugars present. A polysaccharide, consisting of arabinose, xylose, galactose and galacturonic acid units, has been isolated (14) from the hot-water extracts of alkali-pretreated meal. Also a number of polysaccharides have been isolated from soybean meal by successive extractions with water, 0.5% ammonium oxalate, and 0.2, 5, and 15% sodium hydroxide solutions (15). The following sugars have been characterised as hydrolysis products of the alkali extracted hemicelluloses, L-fucose, L-rhamnose, D-xylose, L-arabinose, D-galactose and D-galacturonic acid, but the structural significance of these polysaccharide constituents has not yet been assessed.

Studies of the detailed structure of the polysaccharides of soybeans have been confined to two components of the hulls, namely a galactomannan (16) and a hemicellulose component of the xylan group (18). Whistler and Saarnio (16) extracted soybean hulls with water at 40° and by precipitation with ethanol a galactomannan was obtained in 2% yield. The polysaccharide, which was purified by copper complexing with Fehling's solution, was composed of D-galactose and D-mannose in a ratio of 2.1 : 3. Hydrolysis of the fully methylated derivative yielded 2,3,4,6-tetra-O-methyl-D-galactose (2 moles), 2,3,6-tri-O-methyl-D-mannose (1 mole) and 2,3-di-O-methyl-D-mannose (2 moles), which were characterised as crystalline derivatives. These results showed that D-galactopyranose residues were present exclusively as non-reducing end groups and that D-mannopyranose residues were involved in (1→4) linkages, many of these units also providing branching points through C_6. Periodate oxidation studies provided additional evidence for the proposed structure (I, n = 1) which conforms to the general type of structure (I) of galactomannans isolated from leguminous plants (17).



$$\begin{array}{ccc} \alpha\text{-D-Gal}p & & \alpha\text{-D-Gal}p \\ 1 & & 1 \\ \downarrow & & \downarrow \\ 6 & & 6 \\ \text{-4-}\beta\text{-D-Man}p\text{1-} & [\text{4-}\beta\text{-D-Man}p\text{1}]_n & \text{-4-}\beta\text{-D-Man}p\text{1-} \end{array}$$

(I)

Preliminary results of an investigation of soybean hemicellulose have been reported by Whistler and Sanella (18). Soybean hull holocellulose was extracted with 10% aqueous potassium hydroxide and on neutralisation of the alkaline extracts with acetic acid a hemicellulose A fraction was precipitated in 11% yield. By the addition of ethanol (3 vol.) a further hemicellulose B fraction was isolated and purified by alcohol fractionation to give an electrophoretically homogeneous polysaccharide consisting of a glucuronic acid, D-xylose, L-arabinose, D-galactose and D-glucose moieties. An aldobiouronic acid was separated from the partial acid hydrolysate of this polysaccharide and characterised as 2-O-(4-O-methyl-α-D-glucopyranosyluronic acid)-D-xylose by the formation of its crystalline acetyl derivative.

The cleavage products from the fully methylated polysaccharide included 2-O-methyl-D-xylose, 2,3-di-O-methyl-D-xylose, 2,3-di-O-methyl-D-glucose, 2,3,4,6-tetra-O-methyl-D-galactose and 2,3,5-tri-O-methyl-L-arabinose and 2,3,4-tri-O-methyl-D-xylose in the ratio of 2.4 : 2.0 : 1.5 : 1.5 : 1.0. These results indicated that the polysaccharide was branched with D-galactopyranose, L-arabinofuranose and D-xylopyranose units occupying non-reducing terminal positions with D-xylopyranose and D-glucopyranose units constituting points of branching. It is probable that this polysaccharide is a member of the xylan group of polysaccharides (19), but the detailed structure remains to be fully established.

The aim of the present research is to assess the quantities and nature of the polysaccharide constituents of soybeans in order to obtain basic information that may help to improve the processing of soybean products for food and feeds.

GENERAL METHODS OF INVESTIGATION

General Methods of Investigation

Paper chromatography was carried out on Whatman No. 1 filter paper using the following solvent systems (v/v):-

A) Ethyl acetate - acetic acid - formic acid - water (18:3:1:4)

B) Ethyl acetate - pyridine - water (10:4:3)

C) Ethyl acetate - acetic acid - formic acid - water (18:8:3:9)

D) Butan-1-ol - benzene - pyridine - water (5:1:3:3)

E) Butan-1-ol - ethanol - water (4:1:1)

F) Butan-2-one - light petroleum (b.p. 100-120°) (9:1, half saturated with water)

G) Butan-1-ol - ethanol - water (1:1:1)

H) Butan-1-ol - ethanol - water (4:1:5), upper layer

I) Butan-2-one - acetic acid - water (9:1:1, saturated with boric acid)

J) Butan-2-one - water - ammonia (200:17:1)

Except where otherwise stated chromatograms were run with standard sugars for comparison, air-dried and sprayed with the appropriate reagent.

Chromatographic spray reagents.

(a) p-Anisidine hydrochloride (20) as a 2% solution in butan-1-ol, was the reagent normally employed to detect reducing sugars.

(b) Silver nitrate reagent (21), was used to detect reducing sugars and glycitols.

(c) Aniline Oxalate, as a saturated aqueous solution, was used to detect some methylated reducing sugars.

(d) Periodate/Permanganate reagent (22) was also employed to detect reducing sugars and glycitols.

(e) Hydroxylamine - ferric chloride reagent (23) was used to detect esters and lactones of sugars.

The symbol R_x is used to describe the mobility of a sugar with respect to sugar x on paper chromatograms in the given solvent.

The abbreviation M_G refers to the ionophoretic migration of the sugar with respect to D-glucose in borate buffer, correction being made for electro-endosmotic flow by incorporating a standard of 2,3,4,6-tetra-O-methyl-D-glucose. Similarly M_s refers to the ionophoretic migration of the polyhydroxy compound with respect to sorbitol in molybdate buffer, correction being made for electro-endosmotic flow by incorporating a standard of glycerol.

Paper ionophoresis was carried out on Whatman No. 1 filter paper in borate buffer at pH 10 (24) or molybdate buffer at pH 5 (25). A potential of 350 v was applied over a period of 5-7 hours. The ionophoretograms run in borate buffer were dried and sprayed with a 2% p-anisidine hydrochloride - butan-1-ol solution containing 5% glacial acetic acid, and those run in molybdate buffer were developed with silver nitrate reagent.

Separation of sugars on thick paper.

Whatman 3MM paper sheets, prewashed with water, were used for chromatographic fractionation of sugar mixtures.

COLUMN CHROMATOGRAPHY

Cellulose columns (26).

The cellulose was dry packed and washed with water and butan-1-ol before applying the solvent to be used for the separation of the methylated sugars.

Charcoal-Celite columns (27).

Absorption chromatography on charcoal-Celite mixtures (1:1) was employed to fractionate mixtures of monosaccharides and oligosaccharides. Activated B.D.H. charcoal was washed with boiling distilled water until free from acid. Celite 545 was washed with 50% aqueous hydrochloric acid, filtered , and washed with distilled water until chloride free. The mixture of equal weights of charcoal and Celite was packed as a water slurry into columns and allowed to settle under gravity. Before use the columns were washed with aqueous ethanol and then water. The sugar mixture was allowed to absorb on the column overnight before stepwise or gradient elution with water and water-ethanol mixtures. Fractions were collected automatically and examined by paper chromatography.

Squat charcoal columns (28).

These columns were used for a rapid separation of mixtures of monosaccharides from oligosaccharides. An aqueous slurry of paper pulp was poured into a Buchner funnel to form a cellulose disc (1 cm. thick) on top of which the charcoal was applied as an aqueous slurry. The sugar mixture was applied to the column ; the monosaccharides were eluted with water and then the oligosaccharides were eluted with water containing 35% ethanol.

DEAE-cellulose columns (29).

This anion exchange cellulose was employed for the chromatographic fractionation of polysaccharide mixtures. Diethylaminoethylcellulose was washed alternately with 0.5 N - hydrochloric acid and 0.5 N - sodium hydroxide three times, the turbid supernatant being decanted off each time. The cellulose was then washed with distilled water until free from alkali. A perforated disc was placed at the bottom of the column, then a layer of glass wool, a 1 cm. layer of silver sand and finally a 1 cm. layer of acid-washed Celite. The cellulose was added to the column as a water slurry and packed by the application of slight air pressure. Generation of the cellulose in the phosphate form was performed by eluting the column with 0.5 M - sodium dihydrogen phosphate buffer at pH 6 (2 l.) and then equilibrating it with 0.005 M - sodium dihydrogen phosphate buffer solution (1 l.). The borate form of the cellulose was obtained by elution with 0.2 M - sodium borate (2 l.) and subsequent equilibration with water (2 l.). The cellulose was generated in the acetate form (30) with 0.2 M - sodium acetate (2 l.) and washing free the excess acetate with water (2 l.). The polysaccharide mixture was applied to the column as an aqueous solution and was absorbed on the column overnight. The elution pattern was followed by analysing the fractions by the phenol-sulphuric acid (36) and carbazole methods (37).

DEAE-Sephadex A-25 columns.

This anion exchange cross-linked dextran was employed to fractionate mixtures of acidic oligosaccharides. The DEAE-Sephadex was allowed to swell in an excess of water and washed on a filter with 0.5 N - hydrochloric acid, 0.5 N - sodium hydroxide and finally with water. The Sephadex was added to the column as a slurry in 0.5 N - formic acid and washed free from excess formic acid with water (3 l.). The sugar mixture, as an aqueous solution, was applied to the column and allowed to stand overnight. Neutral sugars were eluted with water and acidic sugars were eluted with formic acid of increasing strength. Fractions were collected automatically and examined chromatographically.

Evaporations were carried out under reduced pressure at or below 40°.

Optical rotations were observed at 18° ± 2°.

Melting-points were determined either in capillary tubes or by using a Kofler hot-stage microscope.

Small-scale hydrolyses.

Samples of polysaccharides (5-10 mg.) and oligosaccharides (1-3 mg.) were totally hydrolysed by heating with N-sulphuric acid (1 - 2 ml.) at 100° for 12 hr. and 3 hr. respectively. The solutions were neutralised with barium carbonate, filtered, the barium ions removed with Amberlite IR-120 (H) resin and the solutions concentrated.

Samples of oligosaccharides (1-3 mg.) were partially hydrolysed by heating with 0.1 N - sulphuric acid (1 ml.) at 100° for 1 - 6 hr. The hydrolysates of neutral oligosaccharides were neutralised directly with Amberlite IR-45 (OH) resin, otherwise the hydrolysates were treated as described above.

Small-scale methylations of oligosaccharides were carried out by shaking the oligosaccharide (0.5 - 2.0 mg.) with methyl iodide (0.2 ml.), dimethylformamide (0.2 ml.) and silver oxide (0.2 g.) at room temperature in the dark for 18 hr. (31). The mixture was filtered and the residue was washed with chloroform. The combined filtrate and washings were evaporated to dryness under 0.03 mm. pressure at room temperature. The methylated oligosaccharides were refluxed in 3% methanolic hydrogen chloride for 10 hr., the solution treated with silver carbonate, filtered, evaporated and the resulting methyl glycosides analysed by gas-liquid partition chromatography.

Gas-Liquid Partition Chromatography (32).

This was carried out using a 'Pye Argon Chromatograph' according to the procedure of Bishop and Cooper (33). Separations were made on the following columns (120 x 0.5 cm.) at gas flow rates of 80 - 100 ml./min. : a) 15% by weight of butan-1,4-diol succinate polyester on dichloro-dimethyl-silane treated Celite (80 - 100 mesh) at 175°, b) 10% by weight of polyphenyl ether [m-bis-(m-phenoxyphenoxy)benzene] on dichloro-dimethyl-silane treated Celite at 200°. The methyl o-methyl glycosides were identified by comparing their retention times (T), relative to methyl-2,3,4,6-tetra-O-methyl-β-D-glucopyranoside, with those of authentic samples run under the same conditions.

Methoxyl content was determined by the semi-micro Zeisel method (34).

Demethylations (35).

The sugar derivative (1-10 mg.) was dissolved or suspended in dry purified methylene chloride (1-2 ml.) and cooled in liquid nitrogen. Boron trichloride (1-2 g.) cooled in the same way was added. The mixture was kept at -80° for 30 min., allowed to warm to room temperature and kept for 16 hr. under anhydrous conditions. Any solvent or boron trichloride was removed under reduced pressure at room temperature. Boric acid was removed by repeated evaporations with methanol.

Nitrogen estimations were carried out by the micro-Kjeldhal method.

Estimations of sugars.

a) Phenol-sulphuric acid method (36).

The sugar content was calculated using a standard curve based on D-galactose.

b) Carbazole method (37).

The uronic anhydride content was calculated using a standard curve based on D-galacturonic acid.

c) Decarboxylation method (38).

This method was found to be more accurate than the carbazole method for determining the uronic anhydride content of polysaccharides.

d) L-Cysteine hydrochloride method (39).

The rhamnose content of oligosaccharides was determined by using this method.

Periodate oxidation of polysaccharides.

The uptake of sodium metaperiodate was followed spectrophotometrically at 222.5 mμ (40).

Borohydride reductions were performed by addition of excess potassium borohydride to an aqueous solution of the sugar, and allowing the solution to stand for 24 hr. Excess borohydride was destroyed and potassium ions removed by the addition of Amberlite IR-120 (H) resin. The solution was filtered, evaporated and the residual boric acid removed by repeated evaporations with methanol.

Aniline derivatives of methylated sugars were prepared by refluxing the sugars (5-15 mg.) with equimolar amounts of freshly distilled aniline in dry ethanol (5-10 ml.). The reaction was performed in the dark for a period of about 30 min. Removal of the solvent left syrups which crystallised and were recrystallised from various solvents.

Aldonolactones of methylated sugars were prepared by oxidation with bromine water. The methylated sugar (5-10 mg.) was dissolved in water (2-3 ml.), bromine (10-20 drops) was added, and this mixture kept in the dark at room temperature for three days. Excess bromine was removed by aeration and the solution neutralised with silver carbonate, filtered, and evaporated to dryness. The residue was extracted with ether and the lactone crystallised by slow evaporation of the ethereal solution.

p-Nitrobenzoate derivative (41).

The methylated sugar (50 mg.) was dissolved in dry pyridine (6 ml.) and treated with p-nitrobenzoyl chloride (200 mg.) for 30 min. at 65-70°. After leaving overnight at room temperature a saturated solution of sodium bicarbonate was added dropwise to the reaction mixture until no further effervescence occurred. Water (5 ml.) was added, and the product extracted with chloroform. The extracts were dried over anhydrous sodium sulphate, filtered, and evaporated to small volume. Addition of light petroleum induced crystallisation of the derivative, which was removed by filtration and recrystallised from methanol.

Purification of organic solvents for column chromatography and for reactions, where specified, were effected by the methods described in the literature (42).

SECTION I

GALACTOMANNANS ISOLATED FROM SOYBEAN HULLS.

SECTION I

DISCUSSION

Galactomannans represent a group of important plant mucilages which occur in the seeds of many plants, particularly the legumes, amounting in some cases to more than 40% of the total seed. They usually occur as mucilages in the endosperm of the seeds from which they may be isolated by extraction with water (43). Their function in the seed appears to be as food reserve polysaccharides since it is known (44) that when the seeds germinate both the mucilage and the endosperm disappear. Galactomannans, which are readily dispersible in water to produce stable solutions of very high viscosity are widely used as thickening and binding agents in the textile, food and paper industries.

Solutions of galactomannans gel in the presence of sodium tetraborate (borax) (45), and alkaline copper salt solutions (46). This complexing ability appears to be due to the <u>cis</u> adjacent hydroxyl groups at C_2 and C_3 in the mannose sugar residues. Cross linkages are formed if the anion $B(OH)_4^-$ (47) or $Cu(OH)_4^{--}$ (48) complexes two adjacent molecular chains leading to the formation of a three dimensional network of didiol - complexes which in turn results in gel formation.

Whistler and Saarnio (16) have shown that soybean hulls contain a galactomannan which may be isolated with cold water, and from methylation studies a structure similar to that of guaran (49) has been proposed. As this research programme was part of an extensive investigation of the polysaccharides of soybeans, the galactomannan fraction from the hulls has been studied in greater detail.

Soybean hulls were extracted with acetone, light petroleum and 80% aqueous ethanol to remove lipids, colouring matter and soluble sugars. The extractive-free hulls were extracted with water at room temperature to give a mixture of polysaccharides, and by subsequent extraction of the hulls with water at 60° a second polysaccharide mixture was obtained. Diethylaminoethylcellulose chromatography of the hot water extracted material indicated the presence in the mixture, of a neutral galactomannan and an acidic polysaccharide fraction which on hydrolysis gave galacturonic acid, galactose, mannose, arabinose, xylose, fucose, rhamnose and two methylated sugars having the chromatographic mobilities of 2-O-methylxylose and 2-O-methylfucose.

The mixtures of polysaccharides isolated from the cold and hot water extracts were fractionated on a large-scale with aqueous cupric acetate (50) which precipitated the contaminating acidic polysaccharides as their insoluble copper salts, and from the resulting supernatant solutions the galactomannan fractions were recovered by precipitation with ethanol. The copper-polysaccharide complexes were decomposed by treatment with ethanolic hydrogen chloride and the polysaccharides dried by solvent exchange.

Galactomannan I, $[\alpha]_D$ + 68°, from the cold water extract, gave on hydrolysis, galactose and mannose in the molar ratio of 1:1.4. Galactomannan II, $[\alpha]_D$ + 26.5°, from the hot water extract, contained galactose and mannose in the molar proportion of 1:2.35. The formation of trace amounts of other sugars was shown to arise from slight contamination with acidic polysaccharides since pure galactomannans could be obtained after chromatography on diethylaminoethylcellulose.

Galactomannan I was methylated with methyl sulphate and sodium hydroxide followed by silver oxide and methyl iodide to yield methylated galactomannan which was fractionated by dissolution in light petroleum-chloroform mixtures, and shown to be essentially homogeneous.

Hydrolysis of methylated galactomannan I, $[\alpha]_D$ + 56°, with sulphuric acid (51) gave as the main cleavage products, 2,3,4,6-tetra-O-methyl-D-galactose, and 2,3,6-tri- and 2,3-di-O-methyl-D-mannose, together with small amounts of 2,3,4,6-tetra-O-methyl-D-mannose and 2,3,6-, 2,4,6-, and 2,3,4-tri-O-methyl-D-galactose, and traces of other sugars which were probably of little structural significance.

Galactomannan II and galactomannan II a (from chromatography on diethylaminoethylcellulose) were methylated as described for galactomannan I and gave methylated derivatives having $[\alpha]_D$ + 12°. These methylated polysaccharides were not examined in detail but gas chromatography of the methyl glycosides formed on methanolysis showed the main cleavage products to be qualitatively similar to those from methylated galactomannan I. Quantitative paper chromatographic estimation (52) of the main hydrolysis products of methylated galactomannans I and II showed the formation of tetra-O-methylgalactose, tri- and di-O-methylmannose in the molar ratios of 2.3 : 1.0 : 2.1 and 1.0: 1.83 : 1.0 respectively. Methylated galactomannan I contained galactose and mannose residues in the same proportions as in the parent polysaccharide. Methylated galactomannan II however, contained a lower proportion of the galactose residues (as its corresponding methylated derivative) than in the parent polysaccharide, and it is probable that some inadvertent fractionation had occurred during the formation of the methylated derivative so that it was not quantitatively representative of the starting material.

The methylation data indicated that the structures of galactomannans I and II were based upon a chain of $(1\longrightarrow 4)$-linked D-mannopyranose units and to C_6 of some of these were attached D-galactopyranose residues. The small amount of tetra-O-methyl-D-mannose in the cleavage products probably arose from end group D-mannose units terminating the main chain. The trimethyl ethers of galactose could have arisen from a small proportion of galactose side chains, or from the presence of a minor polysaccharide contaminant, or more probably from demethylation of the tetra-O-methylgalactose during hydrolysis of the methylated galactomannan. The occurrence of 2,3,6-tri-O-methyl-D-galactose as a hydrolysis fragment of the methylated derivative of the galactomannan from the green palmyra palm nut (53) was attributed to demethylation of the tetra-O-methylgalactose.

Periodate oxidation studies provided additional support to the above formulation. Galactomannan I on oxidation with sodium metaperiodate consumed 1.2 mole (theory required 1.4 mole) of periodate per hexose unit and liberated 0.34 mole (theory required 0.4 mole) of formic acid per sugar residue. Galactomannan II on periodate oxidation consumed 1.1 mole (theory required 1.3 mole) of periodate per hexose unit with a corresponding release of 0.27 mole (theory required 0.3 mole) of formic acid per sugar residue.

Galactomannans I and II were oxidised with sodium metaperiodate and the resulting polyaldehydes were reduced with potassium borohydride to the corresponding polyalcohols which were hydrolysed with dilute mineral acid at room temperature, thereby cleaving the acetal linkages and leaving the glycosidic bonds intact (54). The resulting hydrolysates contained substantial amounts of glycerol and erythritol, originating from the non-reducing terminal units and the (1→4)-linked mannose residues respectively, together with a non-reducing component in approximately 0.5% yield which gave on hydrolysis mannose and erythritol. The isolation of this component was unexpected since the methylation studies had indicated that all the mannose residues possessed free hydroxyl groups on adjacent carbon atoms C_2 and C_3. Incomplete periodate oxidation has, however, been encountered in other galactomannan studies (55) where the hydroxyl groups at C_2 and C_3 of the mannose residues were known to be unsubstituted.

The anomeric configuration of the sugar units in the galactomannan molecule have been determined by the isolation and characterisation of oligosaccharide fragments which were obtained from partial depolymerisation of the polysaccharides.

An investigation on the various methods of partially depolymerising the galactomannan isolated from carob gum (*Orcypria*) indicated that of the following methods 1) mineral acid partial hydrolysis, 2) graded formolysis and 3) graded acetolysis, the latter afforded the maximum yield of oligosaccharide fragments.

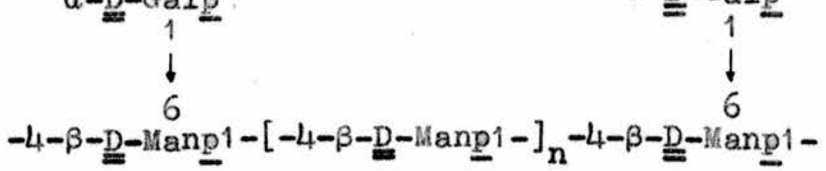
Galactomannans I and II were therefore subjected to graded acetolysis (56) with acetic anhydride, acetic acid and concentrated sulphuric acid (10 : 10 : 1, v/v) and the resulting acetates were deacetylated by treatment with barium methoxide (57). The two galactomannans furnished chromatographically similar mixtures of mono- and oligosaccharides which were combined and fractionated by chromatography on charcoal-Celite followed, where necessary, by partition chromatography on cellulose filter sheets.

The following crystalline oligosaccharides were characterised by comparison with authentic specimens:- 6-*O*-α-D-galactopyranosyl-D-mannose, 4-*O*-β-D-mannopyranosyl-D-mannose, and the polymer-homologous mannotriose, mannotetraose, and mannopentaose. Although *O*-α-D-galactopyranosyl-(1→6)-*O*-β-D-mannopyranosyl-(1→4)-D-mannose was not isolated in a crystalline form, the trisaccharide was recognised by paper chromatography of 6-*O*-α-D-galactopyranosyl-D-mannose and 4-*O*-β-D-mannopyranosyl-D-mannose as partial hydrolysis products and by gas chromatography of methyl glycosides of 2,3,4,6-tetra-*O*-methyl-D-galactose, and 2,3,4- and

2,3,6-tri-*O*-methyl-D-mannose as cleavage products of the methylated derivative.

It is unlikely that small quantities of other oligosaccharides encountered represent fragments of the main galactomannan molecule. Thus 6-*O*-β-D-galactopyranosyl-D-galactose probably originated from a small proportion of polysaccharide contaminant, and the following two series of oligosaccharides were probably formed as artefacts. 4-*O*-β-D-Mannopyranosyl-D-glucose and *O*-β-D-mannopyranosyl-(1→4)-*O*-β-D-mannopyranosyl-(1→4)-D-glucose were probably formed by epimerisation during the process of deacetylation, since glucose was not detected as a constituent of the polysaccharide. 4-*O*-α-D-Mannopyranosyl-D-mannose and *O*-α-D-mannopyranosyl-(1→4)-*O*-β-D-mannopyranosyl-(1→4)-D-mannose were probably formed from the acid-catalysed anomerisation (58) of β-D-mannopyranose residues during acetolysis as demonstrated previously in control experiments (56).

The isolation and identification of the main oligosaccharide fragments have shown that the terminal D-galactopyranose units were joined by (1→6)-α-glycosidic links to a chain of D-mannopyranose units united by links of the (1→4)-β-type, and thus the structures of galactomannans I and II conform to the general structure I which can be formulated for the majority of galactomannans found in the plant kingdom.



$$\begin{array}{ccc} \alpha\text{-D-Gal}p & & \alpha\text{-D-Gal}p \\ 1 & & 1 \\ \downarrow & & \downarrow \\ 6 & & 6 \\ \text{-4-}\beta\text{-D-Man}p\text{1-} & [\text{-4-}\beta\text{-D-Man}p\text{1-}]_n & \text{-4-}\beta\text{-D-Man}p\text{1-} \end{array}$$

I

This structure with single unit side chains of D-galactose residues was in agreement with the findings of Palmer and Ballantyne (59) who investigated guaran using X-ray diffraction methods.

The isolation of the polymer-homologous series of (1→4)-β-linked mannose units up to mannopentaose tended to favour structures with a random distribution of galactose side units along a polymannose chain but it had been shown (53) that the (1→6)-α-linkage between the galactose and mannose residues was much more sensitive to acid attack than the (1→4)-β-mannosidic linkage, thus it would be possible to obtain these oligosaccharide fragments from depolymerisation of a highly ramified regular structure by cleavage of the galactose linkages.

Galactomannans I and II were therefore structurally similar polysaccharides differing only in the degree of substitution of D-galactose residues along the D-mannose backbone. It is noteworthy that subsequent extraction of soybean hulls with alkaline borate solutions furnished a small quantity of a further galactomannan which gave galactose and mannose on hydrolysis in the molar ratio of 1 : 12 (60). It is therefore possible that the isolation of the galactomannan fractions was arbitrary fractionation of a continuous spectrum of closely related galactomannans which contained varying proportions of galactose residues, or the fractions resulted from a definite discontinuity in the proportions of the two sugar units in structurally similar polysaccharides.

Hui and Neukom (61) have investigated the physical properties of guaran from guar seed (62) and locust bean gum from the carob tree (63). The latter was shown to be a mixture of galactomannans, with differing molecular ratios of galactose and mannose, which could be fractionated by dissolution in water. The polymer containing the higher percentage of galactose was found to be more soluble in water due, the authors suggested, to the galactose side units preventing association of the mannan chains. Guaran appeared to be an essentially homogeneous galactomannan. Barry and Halsey (64) have demonstrated that the galactomannan polymer exists in aqueous solution as large aggregates held by random intermolecular hydrogen bonds which can readily be dissociated by elevating the temperature or adding hydrogen bonding agents such as alkali or urea.

Structural studies of galactomannans have largely been confined to the examination of the methyl ethers produced on hydrolysis of the fully methylated polysaccharides. The following table I shows the data derived from the literature and includes the results obtained from the examination of the galactomannans isolated from soybean hulls.

TABLE I

Source	$[\alpha]_D$		Terminal Unit	Chain Unit	Branch Unit	Man/Gal	Ref.
	P/S	Me$^{\underline{d}}$					
Carob (locust) bean (*Ceratonia seliqua L.*)	+9° NaOH	-4° A	Gal*p*1- (1)	-4Man*p*1- (2-4)	6 -4Man*p*1- (1)	3-5	65
Kentucky Coffee bean (*Gymnocladus dioica*)	+29° H_2O	±0° A	Gal*p*1- (1) Man*p*1- (+)	-4Man*p*1- (3)	6 -4Man*p*1- (1)	4	66
Green Palmyra palm nut (*Borassus flabellifer Linn.*)	+8.5° NaOH	+29° C	Gal*p*1- (1)	-4Man*p*1- (1.4)	6 -4Man*p*1- (0.95)	2.4	53
Guar gum (*Cyamopsis tetragonolobus*)	+60° NaOH	+42° C	Gal*p*1- (1)	-4Man*p*1- (1)	6 -4Man*p*1- (1)	2	67
Soybean hulls: galactomannan I	+68°	+56°	Gal*p*1- (2.3)	-4Man*p*1- (1.0)	6 -4Man*p*1- (2.1)	1.4	
galactomannan II	+26.5° H_2O	+12° C	(1.0) Man*p*1- (+)	(1.83)	(1.0)	2.35	

TABLE I (contd.)

Source	$[\alpha]_D$		Terminal Unit	Chain Unit	Branch Unit	Man/Gal	Ref.
	P/S	$\mathrm{Me}^{\underline{d}}$					
Clover (*Trifolium pratense*)	+78° H_2O	+76° C	Galp1- (7)	-4Manp1- (2)	6 -4Manp1- (7)	1.28	55
Lucerne (*var. Provence*)	+118° H_2O	+66° C	Galp1- (4)	-4Manp1- (1)	6 -4Manp1- (4)	1.25	55
Fenugreek (*Trigonella foenum-graecum*)	+70° H_2O	+50° C	Galp1- (5)	-4Manp1- (1)	6 -4Manp1- (5)	1.2	46
Coconut (*Cocos Nucifera*)	-85° NaOH	+25° C	Manp1- (1)	-4Manp1- (11)	-4Galp1- 2 (?)	2	68
			Galp1- (1)	-4Galp1- (3)	(2)		
Lucerne (*Medicago Sativa*)	+89° H_2O	+71° C	Galp1- (1)	-3Galp1- + ? (1)	6 Manp1- 2 + ? (1)	0.5	69

TABLE I (contd.)

Source	$[\alpha]_D$		Terminal Unit	Chain Unit	Branch Unit	Man/Gal	Ref.
	P/S	$Me^{\underline{d}}$					
Seed (Leucaena Glauca)	—	—	Galp1- (4) Manp1- (1)	-4Manp1- (5) -3Manp1- (+) ? { -4Galp1- -3Galp1-	6 -4Manp1- (4)	1.3	70
Trichophyton Granulosum	+71° HCl	+42° C	Manp1- (1) Galf1- (3)	-2Manp1- (5) -6Manp1- (5)	-6Manp1- 2 (2) -4Manp1- 2 (1) Manp1- 3 2 (1)	5.2	71

A = acetone C = chloroform

Figures in parenthesis indicate molar proportions.

The isolation and identification of 4-*O*-β-D-mannopyranosyl-D-mannose, 6-*O*-α-D-galactopyranosyl-D-mannose, *O*-α-D-galactopyranosyl-(1→6)-*O*-β-D-mannopyranosyl-(1→4)-D-mannose and *O*-β-D-mannopyranosyl-(1→4)-*O*-β-D-mannopyranosyl-(1→4)-D-mannose from the hydrolysates of partially depolymerised guar (72), green palmyra palm nut (53), lucerne and fenugreek (73) galactomannans have further substantiated their structures as being similar to the general structure I.

The galactomannans isolated from coconut kernel (68), from lucerne seeds with hot 10% aqueous potassium hydroxide (69) and from the seeds of the tropical plant *Leucaena glauca* (70), differ in some respects to the general formulation for galactomannans found in the plant kingdom.

A unique galactomannan has been isolated from the water-soluble polysaccharides of the dermatophyte *Trichophyton granulosum* (71). Methylation studies have shown that it possessed a highly ramified structure with a main chain of equal numbers of (1→2)- and (1→6)-linked D-mannopyranose units some of which were doubly substituted at positions 2,6 ; 2,4 and 2,3 and that the non-reducing terminal positions were occupied by D-mannopyranose and D-galactofuranose residues. This is the first galactomannan to have been isolated from a micro-organism and it is unique in having all the D-galactose residues in the furanose form.

The function of the galactomannan in nature is still obscure, but the general structure in which the galactose residues are attached, possibly at random, to a chain of mannose residues, could be regarded as a method of storing galactose in the seed. If the galactose is metabolised during the life of the seed, this would account for some, at least, of the variability in the compositions of the galactomannans.

In conclusion it can be stated that the galactomannans isolated from soybean hulls have structures essentially similar to galactomannans isolated from other leguminous seeds.

SECTION I

EXPERIMENTAL

ISOLATION OF GALACTOMANNAN I

Soybeans ("Lindarin" variety) were soaked in acetone for 24 hr., crushed gently to rupture the hulls and separated into hulls, cotyledons and hypocotyls. The hulls were extracted successively in a Soxhlet extractor with acetone, light petroleum (b.p. 60-80°), and ethanol - water (4 : 1) to remove lipids, colouring matter and soluble sugars.

Cold Water Extraction.

The defatted hulls (787 g.) were exhaustively extracted with water (6 x 6 l.) at room temperature, and the resulting solutions were filtered through muslin. The combined filtrates were concentrated to half their original volume and acidified with acetic acid to pH 4.5 in an attempt to precipitate protein (74). The insoluble material (2.2 g., Found : N_2 3.2%) was removed at the "Sharples" centrifuge and the supernatant was neutralised, further concentrated, and poured into ethanol. The precipitate was separated, washed successively with ethanol, acetone and ether and dried in a desiccator to give a polysaccharide mixture (60 g.). Hydrolysis of a sample of the crude polysaccharide gave galacturonic acid, galactose, mannose, arabinose, xylose, fucose, rhamnose and two methylated sugars (probably 2-O-methylxylose and 2-O-methylfucose).

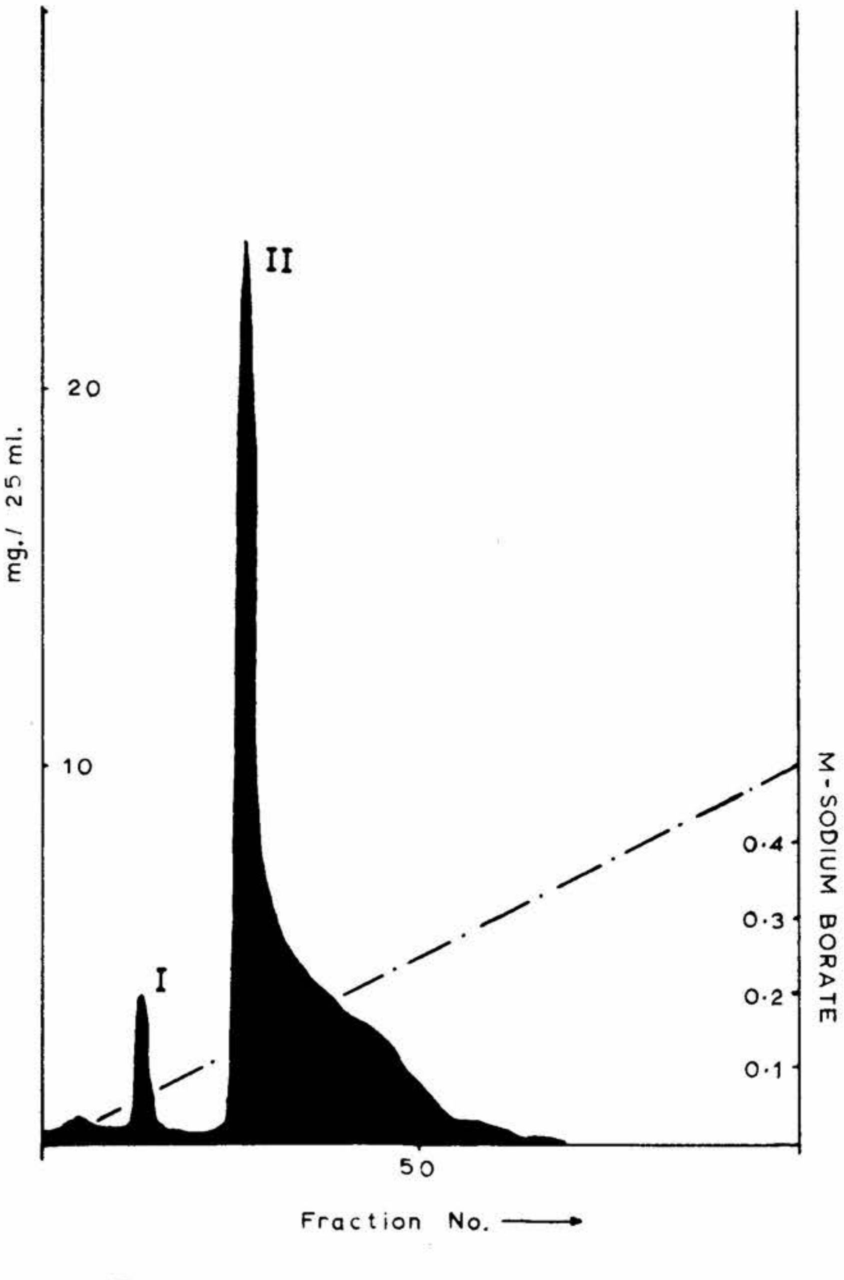
Fractionation with Aqueous Cupric Acetate.

The polysaccharide mixture (59 g.) was dissolved in water (6 l.) and 7% aqueous cupric acetate (600 ml.) added to form a green gelatinous precipitate which was allowed to settle for 4 hr., and then removed at the centrifuge to give insoluble copper complex A. On addition of ethanol (2 vol.) to the supernatant liquor copper complex B separated. Copper complex A was decomposed by treatment with ethanol containing 1% hydrogen chloride, then washed with ethanol, acetone and ether, and dried to yield a polysaccharide (11 g.), $[\alpha]_D + 126°$ (c 1.6 in water), which gave on hydrolysis galacturonic acid, galactose, mannose, arabinose, xylose and traces of fucose, rhamnose and two methylated sugars. Copper complex B similarly afforded galactomannan I as a white fibrous material (11 g.), $[\alpha]_D + 68°$ (c 1.0 in water), (Found : N_2 0.1%) a sample of which gave on hydrolysis galactose, mannose together with a trace of arabinose.

Fractionation of the Acidic Polysaccharide Fraction on Diethylaminoethylcellulose.

A sample (308 mg.) of the polysaccharide isolated from copper complex A was chromatographed on diethylaminoethylcellulose (50 g.) generated in the borate form. The column was eluted with a gradient of water - 0.5 M - sodium borate solution. Fractions (25 ml.) were collected and analysed by

FRACTIONATION of the ACIDIC POLYSACCHARIDE FRACTION on DEAE-CELLULOSE



Eluate: 0·0 → 0·5 M-SODIUM BORATE

the phenol-sulphuric acid and carbazole methods. A plot of polysaccharide concentration per 25 ml. fractions against fraction number is indicated on the accompanying diagram.

Two polysaccharides were detected and the contents of the appropriate tubes were combined, dialysed and concentrated. The polysaccharides were precipitated by the addition of ethanol and dried by solvent exchange. Galactomannan (12 mg.), was eluted with about 0.05 M - sodium borate and gave only galactose and mannose on hydrolysis. The second polysaccharide fraction (126 mg.), $[\alpha]_D$ +102° (c 1.1 in water), was eluted with about 0.1 M- sodium borate and on hydrolysis gave galacturonic acid, galactose, mannose, arabinose, xylose, fucose, rhamnose and two methylated sugars.

ISOLATION OF GALACTOMANNAN II

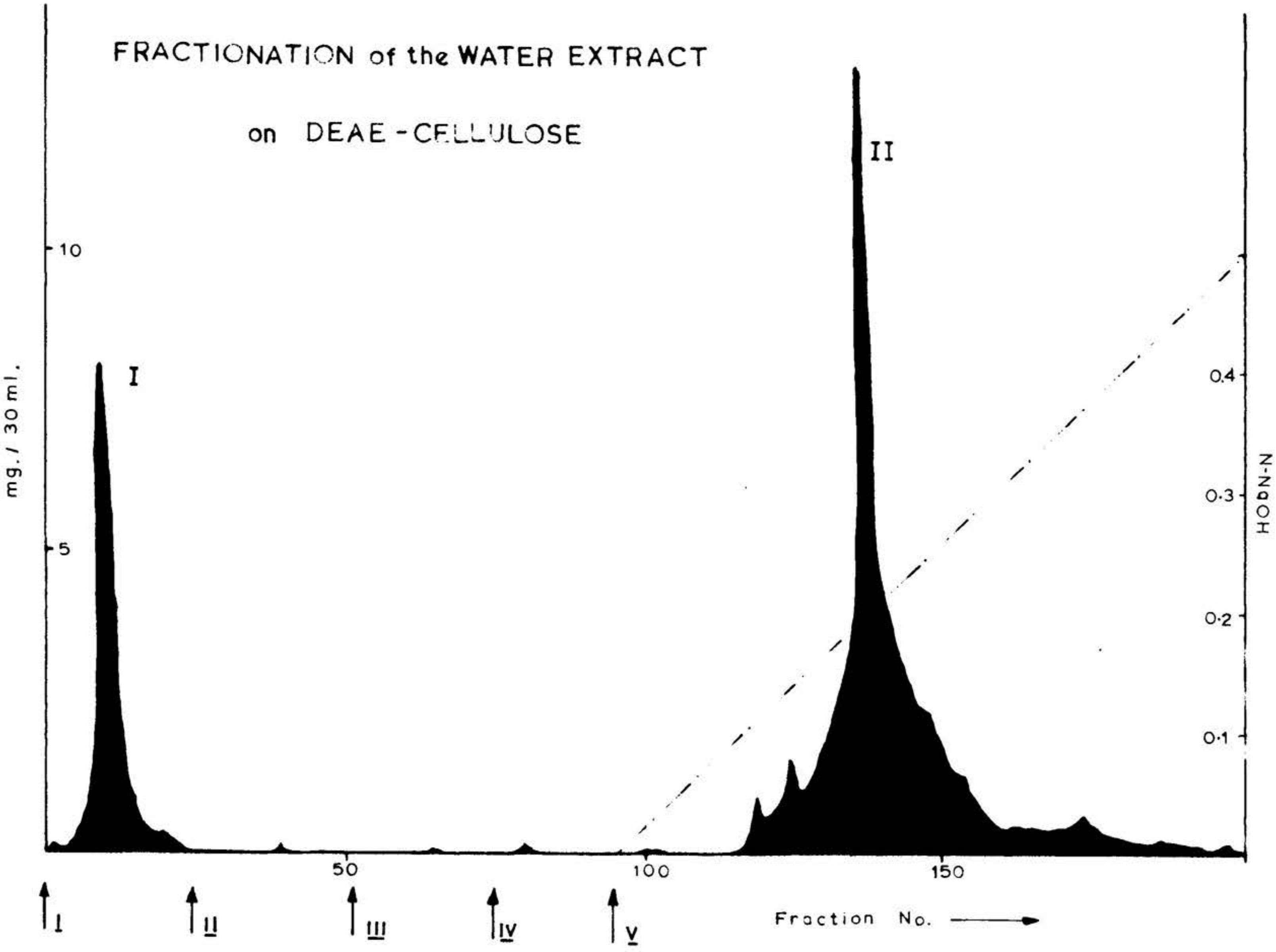
Hot Water Extraction.

The hull residue from the cold water extraction was exhaustively extracted with water (6 x 6 l.) at 60° and the resulting solutions, filtered, concentrated and acidified to pH 4.5 . The insoluble material, (2.8 g.), (Found : N_2 2.9%), was removed at the centrifuge and the supernatant neutralised, concentrated and poured into ethanol. The polysaccharide mixture (45 g.) was dried by solvent exchange. A sample of this material was hydrolysed to give galacturonic acid, galactose, mannose, arabinose, xylose, fucose, rhamnose and two methylated sugars.

Fractionation of the Water Extract on Diethylaminoethylcellulose.

A sample (183 mg.) of the polysaccharide mixture was chromatographed on diethylaminoethylcellulose (30 g.) generated in the phosphate form. The column was eluted successively with 0.025, 0.05, 0.10 and 0.25 M - sodium dihydrogen phosphate buffer at pH 6.1, and then with a gradient of 0.01 - 0.5 N-sodium hydroxide. Fractions (30 ml.) were collected and analysed by the phenol-sulphuric acid method. A plot of polysaccharide concentration per 30 ml. fractions against fraction number is shown on the accompanying diagram.

Two polysaccharide fractions were detected. Galactomannan (37 mg.), $[\alpha]_D$ +30° (c 1.0 in water), was eluted with



FRACTIONATION of the WATER EXTRACT

on DEAE-CELLULOSE

Eluate: I 0·025 M, II 0·05 M, III 0·1 M, IV 0·25 M-PHOSPHATE BUFFER.

V 0·01 - 0·5 N-NaOH.

0.025 M - buffer and gave only galactose and mannose on hydrolysis. The second polysaccharide fraction (197 mg.), which was probably contaminated with cellulosic material, was eluted with sodium hydroxide. Hydrolysis gave, in addition to glucose, galacturonic acid, galactose, mannose, arabinose, xylose, fucose, rhamnose and traces of two methylated sugars.

Fractionation with Aqueous Cupric Acetate.

The polysaccharide mixture (44 g.) was dissolved in water (4 l.) and 7% aqueous cupric acetate (400 ml.) added. The acidic polysaccharide precipitated immediately as an insoluble copper complex A and was removed at the centrifuge. On addition of ethanol (2 vol.) galactomannan copper complex B separated. The complexes were decomposed by treatment with ethanol containing 1% of hydrogen chloride, then washed with ethanol, acetone and ether, and dried in a desiccator.

Copper complex A yielded a polysaccharide (14 g.), $[\alpha]_D$ +75° (c 1.4 in water), which gave on hydrolysis galacturonic acid, galactose, mannose, arabinose, xylose and traces of fucose, rhamnose and two methylated sugars. Copper complex B afforded galactomannan II (5 g.), $[\alpha]_D$ +26.5° (c 1.0 in water), (Found : N_2 0.07%), hydrolysis of which gave galactose and mannose together with traces of arabinose and xylose.

Fractionation of Galactomannan II on Diethylaminoethylcellulose.

A sample (350 mg.) of galactomannan II was dissolved in water (10 ml.) and absorbed on a column (50 g.) of diethylaminoethylcellulose in the phosphate form. The column was eluted successively with 0.025, 0.05, 0.10 and 0.25 M - sodium dihydrogen phosphate buffer at pH 6.1 and then with 0.5 M - sodium chloride.

Galactomannan II a (285 mg.), $[\alpha]_D$ +22° (c 1.0 in water) was eluted with 0.025 M- buffer and gave only galactose and mannose on hydrolysis. A small amount (5 mg.), of a complex acidic polysaccharide was eluted with sodium chloride solution and on hydrolysis gave galacturonic acid, galactose, mannose, arabinose, xylose, fucose, rhamnose and trace amounts of two methylated sugars which were chromatographically identical to 2-O-methylxylose and 2-O-methylfucose.

Quantitative Analysis of the Component Sugars of Galactomannans I and II.

Samples (20 mg.) of the polysaccharides were hydrolysed with 97% formic acid (2 ml.) for 6 hr., at 100°. By repeated evaporation with water the excess formic acid was removed and the resulting formyl esters hydrolysed with N-sulphuric acid (2 ml.) for 1 hr., at 100°. Neutralisation of the sulphuric acid with barium carbonate and removal of the formic acid by evaporation yielded the resulting hydrolysates which were chromatographed on filter sheets in solvent B. The filter-paper strips containing the sugars were extracted with saturated aqueous benzoic acid solution and made up to 10 ml. with distilled water. The galactose solutions were diluted to 40 ml. and the mannose solutions to 100 ml. with concentrated aqueous trichloroacetic acid to give final solutions of these sugars in 4% aqueous trichloroacetic acid.

The colour was developed by adding p-aminodiphenyl reagent (5 ml.) (0.5% in distilled glacial acetic acid containing 0.2% stannous chloride) to samples (1 ml.) of each of the prepared solutions and heating the resulting mixtures for 27 minutes, at 100°C. The solutions were allowed to stand for 18 hr., and their optical densities read at 385 mμ., in a Unicam S.P.600 spectrophotometer. A blank was run concurrently. The ratio of optical densities of mannose to galactose, taking into account the dilution factors, gave a value for the actual ratio of these sugars present in the respective polysaccharides.

Galactomannan I on hydrolysis gave galactose and mannose in the ratio of 1 : 1.4. Galactomannan II contained galactose and mannose in the ratio of 1 : 2.35.

Methylation Studies

Preparation of Methylated Galactomannan I.

A sample of galactomannan I (2.35 g.) in water (10 ml.) was treated with methyl sulphate (30 ml.) and 30% aqueous sodium hydroxide (90 ml.). The methylation mixture was stirred continuously under an atmosphere of nitrogen, at room temperature. Seven further additions of methyl sulphate and aqueous sodium hydroxide were made at 24 hr. intervals (75).

The reaction mixture was heated at 85° for 1 hr., to destroy any unreacted methyl sulphate, then neutralised with dilute sulphuric acid and the resulting solution extracted with hot chloroform (4 x 100 ml.). The combined chloroform extracts were dried over anhydrous sodium sulphate, filtered and evaporated to give a clear glassy solid (3.0 g.) (Found : OMe, 40.7%).

The partly methylated polysaccharide was dissolved in methyl iodide (100 ml.) and silver oxide (6 g.) was added portionwise to the refluxing mixture over a period of 4 hr. (76). The mixture was allowed to reflux for a further 20 hr., the solid was removed by filtration, washed with boiling chloroform and the combined filtrate and washings concentrated. This procedure was repeated twice to yield a methylated product (1.84 g.).

The methylated polysaccharide was fractionated by dissolution in light petroleum (b.p. 60-80°) - chloroform mixtures. The methylated galactomannan (1.75 g.) was refluxed for 10 min. with each solvent mixture (4 x 25 ml.) and the subsequent supernatant solutions were decanted. The dissolved material was recovered by evaporating each solution to dryness. The following fractions were obtained.

Solvent		Weight
% Petroleum	% Chloroform	
100	—	3 mg.
90	10	43.5 mg.
80	20	1,600 mg.
70	30	40.4 mg.

The 80% petroleum fraction was dissolved in chloroform, the solution was poured into excess light petroleum (b.p. 60-80°) and the resulting precipitate was separated, washed with petroleum and dried in vacuo, to give methylated galactomannan I (1.5 g.), $[\alpha]_D$ +56° (<u>c</u> 1.0 in chloroform) (Found: OMe, 44.3%).

<u>Hydrolysis of Methylated Galactomannan I.</u>

The methylated galactomannan (1.36 g.) was treated with 72% sulphuric acid (9 ml.) at 0° for 30 mins. The resulting solution was diluted with water to 216 ml. (3% sulphuric acid) allowed to stand overnight at room temperature and heated at 60° for 18 hours and on a boiling-water bath for 3 hours. The cooled solution was neutralised by passage through a column of Duolite A-4(OH) weak base anion exchange resin and concentrated to a syrupy mixture of sugars.

Separation of the Methylated Sugars.

The mixture of methylated sugars (1.32 g.) was dissolved in dry butan-1-ol (15 ml.) and absorbed on a column of cellulose (60 x 3 cm.). The column was eluted with light petroleum (b.p. 100-120°) - butan-1-ol (7:3, later 1:1 v/v) saturated with water, and butan-1-ol half saturated with water and finally washed with water. Fractions (20 ml.) were collected and examined chromatographically in solvent E. Identical fractions were combined, evaporated to dryness under reduced pressure and the residues purified by dissolution in organic solvents and treatment with charcoal. The solutions were filtered and the residues obtained on evaporation were weighed. The recovery from the column was 91%. A sample of each fraction was methanolysed and the resulting methyl glycosides were examined by gas chromatography on column a and/or column b. The different fractions obtained are shown in table 2.

TABLE 2

Fraction	Wt.(mg.)	R_G in Solvent E	Methyl glycosides $\underline{\underline{T}}$		Content
			Column $\underline{a}$	Column $\underline{b}$	
1	15.4	1.08	0.55		2,3,5-trimethylarabinose(t)
			1.39		2,3,4,6-tetramethylmannose
2	392	0.95	1.8		2,3,4,6-tetramethylgalactose
3	286	0.85	5.1	2.34	2,3,6-trimethylmannose
4	24	0.83	3.21s 3.93vw 4.3w 4.7m		2,3,6-trimethylgalactose
			5.08		2,3,6-trimethylmannose(t)
5	13	0.83	3.21s 3.93vw 4.3w 4.7m		2,3,6-trimethylgalactose
6	7	0.83	3.22s 3.93vw 4.25w 4.7m		2,3,6-trimethylgalactose
			4.0m 4.7s		2,4,6-trimethylgalactose(t)
		0.79	1.94m 1.58s 1.77w		2,3-dimethylarabinose(t)
7	8	0.83	4.19m 4.75s		2,4,6-trimethylgalactose
			3.25s		2,3,6-trimethylgalactose(t)
		0.78	1.95m 1.58s 1.78w		2,3-dimethylarabinose(t)
8	10	0.77	7.5	2.87s 2.62m	2,3,4-trimethylgalactose
			1.94m 1.58s 1.78w	0.64s 0.83w 0.96m	2,3-dimethylarabinose(t)

TABLE 2 (contd.)

Fraction	Wt. (mg.)	R_G in Solvent E	Methyl glycosides T		Content
			Column a	Column b	
9	441	0.68		3.39	2,3-dimethylmannose
10	20	0.62			(?) Me_2 galactose
		0.56			(?) Me_2 mannose
		0.29			(?) Me mannose

(t) = trace

The relative intensities of peaks are indicated by s = strong, m = medium, w = weak and vw = very weak. R_G values relative to 2,3,4,6-tetra-O-methyl-D-glucose.

Examination of Fractions.

Fraction 1.

The syrup (15.4 mg.), $[\alpha]_D$ +2.5° (c 1.4 in water), contained 2,3,4,6-tetra-O-methyl-D-mannose as the major component and 2,3,5-tri-O-methyl-L-arabinose. The gas chromatographic evidence was verified by paper chromatography in solvent F which gave a good separation of the sugars whose detection was further simplified by virtue of the different colours produced by their reaction with aniline oxalate spray reagent, viz. trimethyl arabinose - black, tetramethyl mannose - crimson. Demethylation with boron trichloride gave mannose and a trace of arabinose.

Fraction 2.

The sugar (392 mg.), $[\alpha]_D$ +101° (c 1.0 in water), was examined by gas chromatography and paper chromatography in solvent F, and shown to be 2,3,4,6-tetra-O-methyl-D-galactose giving a characteristic aniline derivative, m.p. and mixed m.p. 197-198°. Demethylation gave only galactose.

Fraction 3.

The sugar, (286 mg.), $[\alpha]_D$ -6.0° (c 1.0 in water), was chromatographically similar to 2,3,6-tri-O-methyl-D-mannose and was characterised by the preparation of the 1,4-di-p-nitrobenzoate derivative, m.p. and mixed m.p. 187-188°. Mannose was obtained on demethylation.

Fraction 4.

The syrup (24 mg.), $[\alpha]_D$ +81.5° (c 1.6 in water) gave galactose and mannose on demethylation. Gas chromatography indicated the presence of 2,3,6-tri-O-methyl-D-galactose with a trace amount of 2,3,6-tri-O-methyl-D-mannose. On oxidation with bromine-water the syrup yielded a lactone, m.p. 97-98° undepressed on admixture with an authentic sample of 2,3,6-tri-O-methyl-D-galactonolactone.

Fraction 5.

The sugar (13 mg.), $[\alpha]_D$ +82° (c 1.0 in water) was chromatographically identical to 2,3,6-tri-O-methyl-D-galactose and gave galactose as the sole product of demethylation. The lactone, recrystallised from ether, gave m.p. and mixed m.p. 98°.

Fraction 6.

The syrup (7 mg.) was shown by gas chromatography, to contain 2,3,6-tri-O-methyl-D-galactose as the major component with trace quantities of 2,4,6-tri-O-methyl-D-galactose and 2,3-di-O-methyl-L-arabinose. Demethylation gave galactose and arabinose.

Fraction 7.

The syrup (8 mg.) gave galactose and arabinose as the products of demethylation. Gas chromatography indicated the presence of 2,4,6-tri-O-methyl-D-galactose with trace amounts of 2,3,6-tri-O-methyl-D-galactose and 2,3-di-O-methyl-L-arabinose. The aniline derivative gave m.p. 174-177° undepressed on admixture with an authentic sample of N-phenyl-2,4,6-tri-O-methyl-D-galactosylamine.

Fraction 8.

The syrup (10 mg.) was examined by gas chromatography and shown to contain 2,3,4-tri-O-methyl-D-galactose with a trace of 2,3-di-O-methyl-L-arabinose. Demethylation gave galactose and arabinose. The aniline derivative failed to crystallise.

Fraction 9.

The sugar (441 mg.), $[\alpha]_D$ -16° (c 1.0 in water) was chromatographically indistinguishable from 2,3-di-O-methyl-D-mannose and gave only mannose on demethylation. Oxidation with bromine-water gave 2,3-di-O-methyl-D-mannonolactone as a syrup. When the lactone was boiled with phenylhydrazine in ethanol there was produced 2,3-di-O-methyl-D-mannonic acid phenylhydrazide m.p. 159-160° which was undepressed on admixture with authentic material.

Fraction 10.

This fraction (20 mg.) contained three sugars.

(a) The sugar (R_G 0.62) gave only galactose on demethylation and was thought to be a dimethylgalactose residue.

(b) The sugar (R_G 0.56) gave only mannose on demethylation and was thought to be a dimethylmannose moiety.

(c) The sugar (R_G 0.29) on demethylation gave mannose and was thought to be a monomethylmannose residue.

These sugars were not examined further.

Preparation of Methylated Galactomannans II and II a.

A sample of the galactomannan II (0.54 g.) was methylated by the addition of eight portions of methyl sulphate (15 ml. each) and 30% aqueous sodium hydroxide (45 ml. each). The partly methylated polysaccharide (0.6 g. as a glass) was isolated by extraction with chloroform and further methylated twice with methyl iodide (20 ml. each) and silver oxide (1 g.) to give methylated galactomannan which was subjected to fractional dissolution. The solvent mixture, light petroleum (b.p. 100-120°) - chloroform (8:2 v/v) was evaporated off leaving methylated polysaccharide which was dissolved in chloroform and precipitated by the addition of light petroleum (b.p. 60-80°) to yield methylated galactomannan II (0.26 g.), $[\alpha]_D$ +12° (c 1.0 in chloroform) (Found: OMe, 44.0%).

Methylation of galactomannan II a (from chromatography on diethylaminoethylcellulose) (0.07 g.) as described for galactomannan II, afforded methylated galactomannan II a (0.03 g.), $[\alpha]_D$ +12° (c 1.0 in chloroform) (Found: OMe, 44.2%).

Gas chromatographic examination of the methanolysis products from Methylated Galactomannans I, II and II a.

A sample (5 mg.) of each of the methylated polysaccharides was heated with 3% methanolic hydrogen chloride for 16 hr. The resulting solutions were neutralised with silver carbonate, filtered and concentrated. Examination of the mixtures of methyl glycosides on columns a and b gave the results shown in table 3.

TABLE 3

Methyl glycosides of -	T in column a Methylated galactomannans			T in column b Methylated galactomannans		
	I	II	II a	I	II	II a
2,3,4,6-tetra-O-methyl-D-mannose	1.41	1.43	1.43	1.31	1.31	1.32
2,3,4,6-tetra-O-methyl-D-galactose	1.78	1.80	1.80	1.58	1.60	1.59
2,3,6-tri-O-methyl-D-mannose	5.07	5.05	5.08	2.34	2.33	2.34
2,3-di-O-methyl-D-mannose	—	—	—	3.40	3.51	3.28

Quantitative Analysis of the Component Sugars of Methylated Galactomannans I and II.

The methylated polysaccharides (50 mg. each) were methanolysed for 16 hr., with 3% methanolic hydrogen chloride (5 ml.) in a sealed tube immersed in a boiling water bath. The methanolic hydrogen chloride was removed by evaporation and the resulting residues treated with 4% aqueous hydrochloric acid (5 ml.) for 6 hr., at 100°. The solutions were neutralised by passage through columns of Amberlite IR-45(OH) resin and the eluates evaporated to yield the resulting hydrolysates which were chromatographed on filter sheets in solvent E. The filter paper strips containing the sugars were extracted for 45 minutes with water (10 ml.) at 80° contained in B24 Quickfit boiling-tubes, and the sugar content of the solutions estimated by the alkaline hypoiodite method (52).

Methylated galactomannan I on hydrolysis yielded tetra-O-methylgalactose, tri- and di-O-methylmannose in the molar ratio of 2.3:1.0:2.1, and these products from methylated galactomannan II were given in the molar ratio of 1.0:1.83:1.0.

Periodate Oxidation Studies.

Periodate Oxidation of Galactomannan I.

Polysaccharide (92.9 mg.) was dissolved in water (90 ml.) and 0.3M - sodium metaperiodate (10 ml.) added. The mixture was allowed to stand in the dark at 20° and at intervals aliquot portions (1 ml.) were withdrawn and diluted to 500 ml. with distilled water and the periodate consumption determined spectrophotometrically. After 23 hr. the periodate consumption was constant at 1.15 mole per mole of sugar residue.

Samples (2 ml.) were removed for the estimation of formic acid by titration with standard sodium hydroxide solution, ethylene glycol (0.5 ml.) having been added to reduce excess periodate. The formic acid release was constant after 48 hr. at 0.34 mole of formic acid per mole of hexose unit.

Periodate Oxidation of Galactomannan II.

Polysaccharide (84.8 mg.) was dissolved in water (90 ml.) and 0.3M - sodium metaperiodate (10 ml.) added. The periodate consumption, measured spectrophotometrically was constant after 19 hr. at 1.08 mole per mole of sugar residue.

Formic acid release was determined by titration with standard sodium hydroxide and after 48 hr. 0.27 mole of formic acid had been produced per hexose residue.

Smith Degradation of Galactomannans I and II.

Samples (100 mg.) of galactomannans I and II were dissolved in water (90 ml.) and 0.3$\underline{\underline{M}}$-sodium metaperiodate (10 ml.) added. The oxidation was allowed to proceed for 96 hr., the excess periodate destroyed with ethylene glycol, and the solutions were treated with potassium borohydride (300 mg.). After standing at room temperature for 72 hr., the solutions were neutralised with dilute acetic acid and dialysed for 48 hr., against running water. The dialysed solutions were evaporated and the residues were dissolved in cold 0.25 $\underline{\underline{N}}$ - sulphuric acid (10 ml.) and left for 30 hr., at room temperature.

The solutions were deionised with Amberlite IR-45(OH) resin and the residues obtained on evaporation were examined chromatographically in solvents, A, D, H and I. The products from the degraded galactomannans I and II were shown to be chromatographically identical. Large quantities of glycerol and erythritol were detected together with a non-reducing component which had an $R_{glucose}$ value of 0.63, 0.68 and 0.75 in solvents A, D and H respectively. The components were resolved on filter sheets in solvent H.

The non-reducing component from galactomannan I (0.6 mg.) and galactomannan II (0.5 mg.) were hydrolysed and chromatographic examination of the hydrolysates in solvents B, D, H and I indicated the presence in both of mannose and erythritol. The glycitol in the hydrolysates was electrophoretically identical to erythritol (M_s 1.0) (threitol, M_s 0.69) in molybdate buffer.

Small-scale Partial Hydrolyses of the Galactomannan isolated from Carob Gum (Orcypria).

(1) With 0.5N - sulphuric acid.

The polysaccharide (0.5 g.) was heated in 0.5N - sulphuric acid (25 ml.) on a water bath at 100°. Samples (1 ml.) were withdrawn at intervals, neutralised with barium carbonate, treated with Amberlite IR-120(H) resin, were concentrated and examined by chromatography in solvents A and B. The results indicated the maximum production of the oligosaccharides mannobiose, galactosylmannose and mannotriose after 4 hr.

(2) Formolysis.

The polysaccharide (0.5 g.) was heated in 45% formic acid (20 ml.) on a boiling water bath and at intervals, samples (2 ml.) were taken and the excess formic acid removed by repeated evaporation with water. The resulting formyl esters were hydrolysed with 0.25N - sulphuric acid (5 ml.) for 10 min. at 100°. Neutralisation of the sulphuric acid with barium carbonate and removal of the formic acid by evaporation yielded hydrolysates which were examined by chromatography in solvents A and B. Mannobiose, galactosylmannose and mannotriose were produced in maximum yield after 1.5 hr.

(3) Acetolysis followed by Deacetylation.

Galactomannan (0.5 g.) was added in small portions to an ice-cooled acetolysis mixture [acetic acid (5.2 ml.), acetic anhydride (5.2 ml.) and concentrated sulphuric acid (0.52 ml.)]. The solution was kept at room temperature and samples (2 ml.) were withdrawn every 24 hr., poured into ice-water (10 ml.) and the solutions were adjusted to pH 4-5 by the addition of sodium hydrogen carbonate. Sugar acetates were extracted with chloroform and the solutions dried over anhydrous sodium sulphate and concentrated. The resulting syrups were dissolved in methanol (10 ml.) and chloroform (5 ml.), 0.5N - barium methoxide (5 ml.) was added and the solutions were allowed to stand for 24 hr. at 0°. Dilute sulphuric acid was added to pH 7, barium sulphate was removed by centrifugation and the solutions were concentrated and examined by chromatography in solvents A and B. The results indicated that the optimum time for the formation of oligosaccharides was after acetolysis for 72 hr.

Chromatographic evidence indicated that of the three methods employed to depolymerise the galactomannan, the process of graded acetolysis followed by deacetylation furnished the maximum production of oligosaccharides with the least amount of total hydrolysis.

ACETOLYSES OF GALACTOMANNANS I and II.

Galactomannans I and II were subjected to graded acetolyses and the products of depolymerisation fractionated on charcoal-Celite. Several oligosaccharides have been characterised.

A trial acetolysis on the galactomannan isolated from carob gum indicated that a reaction period of 72 hours was required for the maximum production of oligosaccharides.

Acetolysis of Galactomannan I followed by Deacetylation.

The galactomannan I [5.08 g., $[\alpha]_D$ +68° (c 1.0 in water)] was added slowly with stirring to a mixture of acetic acid (50 ml.), acetic anhydride (50 ml.), and concentrated sulphuric acid (5.0 ml.) and the mixture kept at room temperature for 72 hours. The mixture was poured into ice-water (300 ml.) and sodium hydrogen carbonate was added gradually to pH 4-5. The precipitated sugar acetates were separated and dissolved in chloroform (100 ml.) and the aqueous solution extracted with chloroform (4 x 100 ml.). The combined chloroform solutions were washed with sodium hydrogen carbonate solution until free from acid, dried over anhydrous sodium sulphate, filtered through a sintered glass funnel, and concentrated to a syrup (ca. 8 g.).

The acetates were deacetylated by the catalytic barium methoxide method. Barium methoxide (50 ml. of 0.5N solution in methanol) was added to the syrupy acetates in chloroform (10 ml.) and methanol (20 ml.), and the mixture kept at 0° for 24 hours.

The mixture was poured into water (200 ml.), barium ions were precipitated by neutralisation with dilute sulphuric acid to pH 7.0, and the filtrate was passed through Amberlite IR-120(H) and Duolite A-4(OH) resins and concentrated to a syrup (3.18 g.). The syrup was examined chromatographically in solvents A and B.

Acetolysis of Galactomannan II followed by Deacetylation.

The galactomannan II, [2.3 g., $[\alpha]_D$ +26.5° (c 1.0 in water)], was treated for 72 hours with an acetolysis mixture (52.5 ml.) in the manner already described. The solution was poured into ice-water (150 ml.) and sodium hydrogen carbonate added gradually to pH 4-5. The precipitated sugar acetates were separated and dissolved in chloroform (50 ml.) and the aqueous solution extracted with chloroform (4 x 50 ml.). The combined chloroform solutions were washed with sodium hydrogen carbonate solution until free from acid, dried over anhydrous sodium sulphate, filtered through a sintered-glass funnel and concentrated to a syrup (ca 4 g.).

Barium methoxide (25 ml. of 0.5N solution in methanol) was added to a solution of the acetates in chloroform (5 ml.) and methanol (10 ml.), and the mixture was kept at 0° for 24 hours. The solution was deionised and concentrated to a syrup (1.68 g.) which was examined by paper chromatography in solvents A and B.

Fractionation of Oligosaccharides on Charcoal-Celite.

The syrups obtained on acetolyses of the galactomannans were examined by paper chromatography in solvents A and B and shown to contain mixtures of the same components including mannose, galactose, mannobiose, mannotriose, galactosylmannose and other oligosaccharides.

The combined mixtures of sugars (4.77 g.) were dissolved in water (20 ml.) and added to a column (9 cm. x 7 cm.) of charcoal-Celite (1:1. 150 g. each). The sugars were eluted successively with water (3 l.) and water containing increasing proportions of ethanol (0 - 25% ethanol). Fractions (25 ml.) were collected in test-tubes and every tenth was evaporated to dryness and examined chromatographically in solvents B and G. Identical fractions were combined and evaporated to dryness. Where necessary further fractionation was carried out by chromatography on Whatman filter sheets using an appropriate solvent system.

Elution with water yielded a mixture of monosaccharides (1.74 g., galactose, mannose, with traces of arabinose and xylose) together with a trace of mannobiose. The fractions obtained are shown in table 4.

TABLE 4

Fraction	% EtOH in Eluant	Weight (mg.)	Component Sugars	$R_{glucose}$ (in Solvent B)	Relative Amounts
1	1.0 - 5.0	369	O_1	0.50	
2	1.0 - 5.0	82	O_1 O_2 O_3 O_4 O_5	0.50 0.66 0.42 0.35 0.25	++++ ++ ++++ trace +
3	1.0 - 5.0	35	O_2 O_3 O_4	0.66 0.42 0.35	++ ++++ +
4	5.0	59	O_2 O_3 O_4 O_6	0.66 0.42 0.35 0.17	trace ++++ + trace
5	5.0	171	O_3 O_4 O_6	0.42 0.35 0.17	trace trace ++++
6	5.0 - 7.5	205	O_6	0.17	
7	5.0 - 7.5	110	O_6 O_7	0.17 0.22	++++ +++
8	5.0 - 7.5	61	O_7 O_6, O_8 O_9	0.22 0.16 0.12	+ ++++ trace
9	5.0 - 7.5	53	O_7 O_8 O_9	0.22 0.16 0.12	trace ++++ +
				$R_{glucose}$ (in Solvent G)	
10	7.5-12.5	210	O_{10} O_{11}, O_{11a}	0.32 0.41	+++ ++

Table 4 (contd.)

Fraction	% EtOH in Eluant	Weight (mg.)	Component Sugar	$R_{glucose}$ (in Solvent G)	Relative Amounts
11	12.5-15.0	74	O_{11}, O_{11a}	0.41	
12	12.5-15.0	250	O_{12} + Complex mixture of faster moving oligosaccharides	0.04	+ ++++
Residue	25.0	950	O_{12} + Complex mixture of oligosaccharides	0.04	

Identification and Characterisation of Oligosaccharides.

Oligosaccharide 1 (O_1) 376 mg.
$R_{glucose}$ = 0.50 in solvent B
M_G = 0.54

The sugar gave only mannose on hydrolysis and was chromatographically and electrophoretically indistinguishable from authentic 4-*O*-β-*D*-mannopyranosyl-*D*-mannose. The disaccharide was recrystallised from ethanol containing a trace of water and the acicular crystals had m.p. and mixed m.p. 205°, $[\alpha]_D$ -7.5° (equil.)(*c* 5.1 in water), and gave an X-ray powder photograph identical with that given by 4-*O*-β-*D*-mannopyranosyl-*D*-mannopyranose. The methanolysis products from the methylated disaccharide were examined by gas chromatography and were shown to contain methyl glycosides of 2,3,4,6-tetra- and 2,3,6-tri-*O*-methyl-*D*-mannose.

Oligosaccharide 2 (O_2) 8 mg.
$R_{glucose}$ = 0.66 in solvent B
M_G = 0.57

The sugar, $[\alpha]_D$ +66° (*c* 0.68 in water) gave mannose on hydrolysis, and was chromatographically and electrophoretically indistinguishable from 4-*O*-α-*D*-mannopyranosyl-*D*-mannose from ivory nut mannan (77). The methanolysis products from the methylated disaccharide were examined by gas chromatography and the main components had retention times of methyl glycosides of 2,3,4,6-tetra- and 2,3,6-tri-O-methyl-D-mannose.

Oligosaccharide 3. (O$_3$) 40 mg.

$R_{glucose}$ = 0.42 in solvent B

M_G = 0.65

The disaccharide, which gave galactose and mannose on hydrolysis, was recrystallised from ethanol - water to give 6-O-α-D-galactopyranosyl-D-mannose which was identified by m.p. and mixed m.p. 201°, $[\alpha]_D$ + 122° (equil.) (c 1.0 in water), and by an X-ray powder photograph. The methanolysis products from the methylated disaccharide were examined by gas chromatography and found to contain components having the retention times of 2,3,4,6-tetra-O-methyl-D-galactose and 2,3,4-tri-O-methyl-D-mannose.

Oligosaccharide 4 (O$_4$) 1 mg.

$R_{glucose}$ = 0.35 in solvent B

M_G = 0.36

The sugar gave mannose and glucose on hydrolysis and was chromatographically and electrophoretically indistinguishable from 4-O-β-D-mannopyranosyl-D-glucose.

Oligosaccharide 5 (O_5) 2 mg.

$R_{glucose}$ = 0.25 in solvent B
M_G = 0.80

The sugar gave only galactose on hydrolysis and was chromatographically and electrophoretically indistinguishable from 6-*O*-β-*D*-galactopyranosyl-*D*-galactose but distinguishable from 4-*O*-β-*D*-galactopyranosyl-*D*-galactose.

Oligosaccharide 6 (O_6) 254 mg.

$R_{glucose}$ = 0.17 in solvent B
M_G = 0.50

The sugar yielded only mannose on hydrolysis and had chromatographic and electrophoretic mobilities identical with those of *O*-β-*D*-mannopyranosyl-(1 → 4)-*O*-β-*D*-mannopyranosyl-(1 → 4)-*D*-mannose. Crystallisation occurred readily from *iso*propanol - water ; the plate-shaped crystals exhibited m.p. (sealed tube) 131-132°, m.p. and mixed m.p. 166-168° (slow heating), $[\alpha]_D$ -15° → -20° (equil.)(*c* 1.1 in water) and X-ray powder photographs were identical with those of *O*-β-*D*-mannopyranosyl-(1 → 4)-*O*-β-*D*-mannopyranosyl-(1 → 4)-*D*-mannose trihydrate.

The methanolysis products from the methylated trisaccharide were examined by gas chromatography and were shown to contain components having the retention times of 2,3,4,6-tetra- and 2,3,6-tri-*O*-methyl-*D*-mannose.

Oligosaccharide 7 (O_7) 12 mg.

$R_{glucose}$ = 0.22 in solvent B

M_G = 0.60

The sugar gave only mannose on hydrolysis and showed a chromatographic mobility and electrophoretic mobility identical with that of authentic O-α-D-mannopyranosyl-(1→4)-O-β-D-mannopyranosyl-(1→4)-D-mannose from ivory nut mannan (77). The trisaccharide crystallised from ethanol - water and had m.p. and mixed m.p. 224°, $[\alpha]_D$ +41° (equil.)(c 1.2 in water), and gave an X-ray powder photograph identical with that obtained from an authentic sample of O-α-D-mannopyranosyl-(1→4)-O-β-D-mannopyranosyl-(1→4)-D-mannopyranose. Partial acid hydrolysis of the derived glycitol (borohydride reduction) gave mannose and 4-O-α-D-mannopyranosyl-D-mannose. The methanolysis products from the methylated trisaccharide were examined by gas chromatography and were shown to contain methyl glycosides of 2,3,4,6-tetra- and 2,3,6-tri-O-methyl-D-mannose.

Oligosaccharide 8 (O_8) 17 mg.

$R_{glucose}$ = 0.16 in solvent B

M_G = 0.48

The syrup, $[\alpha]_D$ +31° (c 0.9 in water), gave on hydrolysis galactose and mannose in the proportions of 1:2. Partial acid hydrolysis of the syrup yielded galactose, mannose, mannobiose and 6-O-α-D-galactopyranosyl-D-mannose ; partial acid hydrolysis of the derived glycitol (borohydride reduction) gave galactose, mannose and 6-*O*-α-D-galactopyranosyl-D-mannose. The syrup was methylated and the methanolysis products from the methylated sugar were examined by gas chromatography and were shown to contain components having the retention times of 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,3,4-tri- and 2,3,6-tri-*O*-methyl-D-mannose. This evidence indicated a trisaccharide with the structure *O*-α-D-galactopyranosyl-(1→6)-*O*-β-D-mannopyranosyl-(1→4)-D-mannose.

Oligosaccharide 9 (O_9) 5 mg.

$R_{glucose}$ = 0.12 in solvent B

M_G = 0.38

The sugar, $[\alpha]_D$ -10° (*c* 0.4 in water), gave mannose and glucose on hydrolysis and showed a chromatographic and electrophoretic mobility identical with that of authentic *O*-β-D-mannopyranosyl-(1→4)-*O*-β-D-mannopyranosyl-(1→4)-D-glucose. Partial acid hydrolysis of the sugar yielded glucose, mannose, 4-*O*-β-D-mannopyranosyl-D-mannose and 4-*O*-β-D-mannopyranosyl-D-glucose; partial hydrolysis of the derived glycitol (borohydride reduction) gave mannose and mannobiose.

Oligosaccharide 10 (O_{10}) 20 mg.

$R_{glucose}$ = 0.32 in solvent G

M_G = 0.47

The sugar was crystallised from ethanol-water and had m.p.

and mixed m.p. 231-232°, $[\alpha]_D$ -30° (10 minutes) → -28° (equil.) (*c* 1.5 in water), and had an X-ray powder photograph identical to that of an authentic sample of *O*-β-*D*-mannopyranosyl-(1→4)-*O*-β-*D*-mannopyranosyl-(1→4)-*O*-β-*D*-mannopyranosyl-(1→4)-*D*-mannose. The sugar gave only mannose on hydrolysis and after partial acid hydrolysis chromatographically showed the homologous series, mannose, mannobiose, mannotriose and unchanged tetrasaccharide. Borohydride reduction followed by partial acid hydrolysis yielded the homologous series up to mannotriose. Gas chromatography indicated the presence of the methyl glycosides of 2,3,4,6-tetra- and 2,3,6-tri-*O*-methyl-*D*-mannose in the product of methanolysis of the methylated tetrasaccharide.

Oligosaccharide 11 and 11a 80 mg.

$R_{glucose}$ = 0.41 in solvent G

(O_{11}) M_G = 0.47

(O_{11a}) M_G = 0.53

This fraction was chromatographically similar in solvents B and G to *O*-α-*D*-mannopyranosyl-(1→4)-*O*-β-*D*-mannopyranosyl-(1→4)-*O*-β-*D*-mannopyranosyl-(1→4)-*D*-mannose from ivory nut mannan (77). Electrophoresis indicated the presence of two sugars the value of 0.53 agreeing with that of the authentic sample of *iso*mannotetraose. The syrup on hydrolysis yielded galactose and mannose and after partial acid hydrolysis chromatographically showed galactose, mannose, mannobiose, *iso*mannobiose, mannotriose, *iso*mannotriose, galactosylmannose and unchanged tetrasaccharide.

Borohydride reduction followed by partial acid hydrolysis yielded, chromatographically, the same products as above except for the unchanged tetrasaccharide. There was some evidence therefore, for the presence of isomannotetraose (O_{11a}) in this fraction together with a tetrasaccharide (O_{11}) which contained galactose and mannose.

Oligosaccharide 12 (O_{12}) 15 mg.

$R_{glucose}$ = 0.04 in solvent G

M_G = 0.41

The sugar was crystallised from ethanol-water and had $[\alpha]_D$ -30° (c 0.98 in water); no m.p. (in excess of 290°) could be recorded. An X-ray powder photograph of the pentasaccharide was identical with that given by O-β-D-mannopyranosyl-(1⟶4)-[O-β-D-mannopyranosyl-(1⟶4)]$_3$-D-mannose, and substantially different from the lower homologue mannotetraose. The methanolysis products from the methylated pentasaccharide were examined by gas chromatography and shown to contain methyl glycosides of 2,3,4,6-tetra- and 2,3,6-tri-O-methyl-D-mannose.

SECTION II

POLYSACCHARIDES ISOLATED FROM SOYBEAN COTYLEDON MEAL.

SECTION II

DISCUSSION

The high protein content of soybean cotyledon meal presented considerable difficulties to the isolation of the polysaccharide components in a relatively pure state. Extraction of the meal with boiling ethanol - water removed most of the soluble sugars, fructose, sucrose, raffinose and stachyose (78) and subsequent extraction of the meal with water alone was found to remove only small amounts of protein since the following 0.5% aqueous ammonium oxalate and 0.2% and 5% aqueous sodium hydroxide extracts (extraction scheme A) were highly contaminated with protein. The polysaccharide components from these extracts were similar in giving rise on hydrolysis to the same mixtures of sugars containing galacturonic acid, galactose, arabinose, xylose, fucose, ribose, rhamnose and methylated sugars together with traces of mannose and glucose.

A more satisfactory extraction procedure (extraction scheme B) involved an extraction of the meal with cold very dilute alkali to solubilise most of the protein (13) and although the swollen meal was rather difficult to handle at this stage subsequent extractions with 0.5% aqueous ammonium oxalate and 5% aqueous sodium hydroxide gave polysaccharide preparations containing relatively little contamination with protein. The polysaccharide components of these extracts contained the same constituent sugars but in varied proportions. The 0.5% aqueous ammonium oxalate extract M2 gave on hydrolysis galacturonic acid, galactose, arabinose, xylose, fucose, ribose, rhamnose, 2-_O_-methylxylose, 2-_O_-methylfucose together with traces of mannose and glucose ; this extract was examined in greater detail.

Fractionation of the polysaccharide extract M2 on diethylaminoethylcellulose, generated in the phosphate and acetate forms, showed that the polysaccharide system was heterogeneous, containing a number of acidic polysaccharide components. The major fractions possessed the same constituent sugars as the parent polysaccharide complex, except for ribose which appeared in some way to have been irreversibly absorbed on the cellulose.

An attempt to fractionate the polysaccharide complex M2 with cupric acetate (50), calcium chloride (79) and cetavlon (80) procedures failed to effect any significant separation. Although some measure of heterogeneity was apparent in extract M2 it was probable that the component polysaccharide fractions containing identical constituent sugars also contained many structural features in common. Since there was no evidence for the presence of entirely different types of polysaccharides present in the polysaccharide complex M2 various methods of degrading the complex were investigated in order to obtain conditions for the optimum production of simpler fragments, including oligosaccharides which could be isolated and characterised, thereby furnishing information on the structural features of the polysaccharide and the types of linkages likely to be present in the structure.

Mild acid hydrolysis of a 2% solution of the polysaccharide complex M2 in 0.02N - sulphuric acid resulted in the rapid release of arabinose and fucose residues suggesting that these sugars were probably present in the molecule, in part, as peripheral units and that the arabinose residues were present in the furanose form. Galactose and xylose were also released as cleavage products at a later stage in the hydrolysis but oligosaccharides were not detected in the hydrolysate at any time during the hydrolysis.

More drastic partial hydrolysis conditions, N-sulphuric acid for 4 hr. at 100°, in a 2% solution of the polysaccharide fraction M2 afforded degraded polysaccharides and a mixture of acidic oligosaccharides containing galacturonic acid and rhamnose moieties which were subsequently characterised in a later experiment. Only traces of neutral oligosaccharides were obtained under these hydrolysis conditions.

The polysaccharide complex M2 was subjected to graded acetolysis with acetic acid, acetic anhydride and sulphuric acid (10:10:1, v/v) and deacetylation of the acetolysis products furnished a mixture of neutral oligosaccharides containing, fucose, xylose, galactose and rhamnose, and included components with the chromatographic mobilities of 2-O-α-L-fucopyranosyl-D-xylose and 2-O-β-D-galactopyranosyl-D-xylose.

A modified procedure was used for the isolation of further quantities of polysaccharides from soybean cotyledon meal. The isolation of the polysaccharide fractions in two stages involving extraction with 0.5% aqueous ammonium oxalate and with 5% aqueous sodium hydroxide (extraction scheme B) was replaced by a single extraction procedure using 2% aqueous ethylenediaminetetraacetic acid disodium salt (extraction scheme C) which removed most of the polysaccharide from the cotyledon meal previously pretreated with boiling ethanol-water to remove soluble sugars and 0.2% aqueous sodium hydroxide to solubilise the major part of the protein.

The soybean meal ethylenediaminetetraacetic acid disodium salt extract ME2 was subjected to a detailed examination. The fraction had a specific rotation of +41° and gave on hydrolysis galacturonic acid, galactose, mannose, glucose, arabinose, xylose, fucose, rhamnose, 2-*O*-methylxylose and 2-*O*-methylfucose. Fractionation of the extract on a diethylaminoethylcellulose column generated in the phosphate form gave an elution pattern identical to that given by the ammonium oxalate extract M2 fractionated on the same type of column.

Two major and three minor fractions were isolated and partial hydrolysis of the five fractions with 0.5*N* - sulphuric acid gave a mixture of neutral and acidic sugars which were separated with diethylaminoethyl-Sephadex. 4-*O*-β-*D*-Galactopyranosyl-*D*-galactose and the corresponding trisaccharide were detected as neutral components from all but the second major fraction which yielded these fragments only on very mild hydrolysis with 0.1*N* - sulphuric acid. The two major polysaccharide components isolated from the column differed also in the relative amounts of their acidic and neutral component sugars. The first fraction with a uronic acid content of 16% contained galactose and arabinose as the principal sugars with smaller amounts of xylose, whereas the second fraction contained xylose in much greater amount than galactose and arabinose and had a uronic acid content of 36%. These observations suggested that the two major polysaccharide fractions from the column differed in their proportions of side chains and, in particular, the length of the polygalactose side units. These differences could explain their separation on diethylaminoethylcellulose since Neukom (81) has shown that the extent of absorption of pectic substances on this ion exchange cellulose depended on the degree of esterification, the degree of polymerisation and the proportion of side chains.

The acidic oligosaccharides from the partial hydrolysis of the two major polysaccharide fractions were chromatographically identical and had chromatographic mobilities similar to the acidic sugars isolated from partial hydrolysis (N - sulphuric acid) of the 0.5% aqueous ammonium oxalate M2 extract. Similarly the products of graded acetolysis of the two major polysaccharide fractions furnished chromatographically similar mixtures of sugars to that isolated from the graded acetolysis of the aqueous ammonium oxalate M2 extract.

Since it had been shown that the aqueous ammonium oxalate polysaccharide complex M2 and the aqueous ethylenediaminetetraacetic acid disodium salt polysaccharide complex ME2 furnished similar mixtures of sugars on partial depolymerisation, samples of the latter were partially hydrolysed in large enough quantities to enable oligosaccharide fragments to be isolated and characterised.

Partial hydrolysis of the polysaccharide complex ME2 in 0.5N - sulphuric acid yielded a small quantity of insoluble degraded polysaccharide, a degraded polysaccharide precipitated from the hydrolysate by the addition of acetone and a mixture of mono- and oligosaccharides. Hydrolysis of a sample of the insoluble degraded polysaccharide gave galacturonic acid, glucose, xylose, rhamnose, 2-*O*-methylxylose, 2-*O*-methylfucose and traces of galactose and mannose; the precipitated degraded polysaccharide yielded galacturonic acid, xylose, rhamnose, 2-*O*-methylxylose, 2-*O*-methylfucose and a trace of galactose.

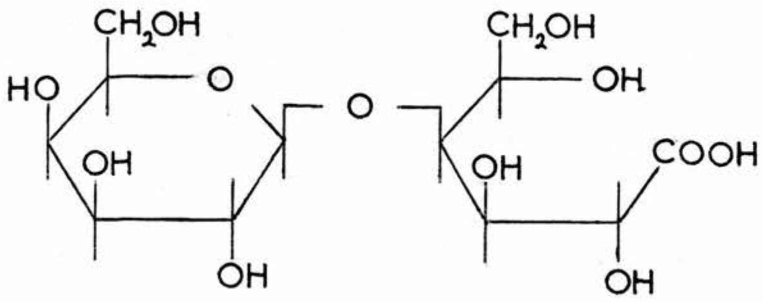
The oligosaccharide components were separated from the monosaccharides on charcoal and then sub-divided into neutral and acidic fractions on diethylaminoethyl-Sephadex. Paper chromatographic examination of the acidic oligosaccharides showed them to be chromatographically similar to those isolated and characterised in a later experiment. The neutral oligosaccharides were fractionated on a charcoal-Celite column followed, where necessary, by partition chromatography on cellulose filter sheets.

2-*O*-Methylxylose and 2-*O*-methylfucose were identified as products of the partial hydrolysis together with the main products which were members of a homologous series II of galactose-containing oligosaccharides.

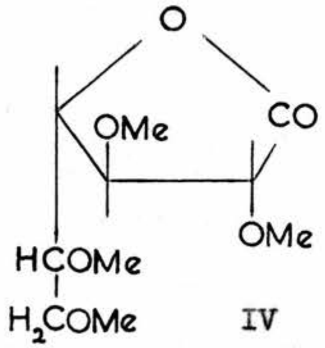
$$\beta\text{-}\underline{D}\text{-Gal}\underline{p}\text{-}[(1\longrightarrow 4)\text{-}\beta\text{-}\underline{D}\text{-Gal}\underline{p}]_n\text{-}(1\longrightarrow 4)\text{-}\underline{D}\text{-Gal}\underline{p}$$

II

The first member was a disaccharide which crystallised from ethanol-water and was identified by comparison (m.p. and mixed m.p., specific rotation and X-ray powder photograph) with an authentic specimen of 4-O-β-D-galactopyranosyl-D-galactose (II, n = 0). As the structure of the disaccharide (II, n = 0) had been based on lead tetraacetate oxidation studies (82) the oligosaccharide was subjected to methylation studies in order to substantiate the formulation. The cleavage products of the fully methylated disaccharide were characterised as 2,3,4,6-tetra- and 2,3,6-tri-O-methyl-D-galactose. The disaccharide was oxidised with bromine-water to the corresponding acid III followed by methylation and hydrolysis to yield 2,3,4,6-tetra-O-methyl-D-galactose and 2,3,5,6-tetra-O-methyl-D-galactonolactone IV. These results confirmed the structure of the disaccharide as 4-O-β-D-galactopyranosyl-D-galactose, (II, n = 0).



III



IV

The second member of the series was a trisaccharide which on partial hydrolysis gave galactose and 4-O-β-D-galactopyranosyl-D-galactose. The methylated sugar on methanolysis yielded the methyl glycosides of 2,3,4,6-tetra- and 2,3,6-tri-O-methyl-D-galactose. The cleavage products of the methylated oxidised trisaccharide were shown by gas and paper chromatography to be 2,3,4,6-tetra- and 2,3,6-tri-O-methyl-D-galactose together with 2,3,5,6-tetra-O-methyl-D-galactonolactone, proving the structure to be O-β-D-galactopyranosyl-(1⟶4)-O-β-D-galactopyranosyl-(1⟶4)-D-galactose (II, n = 1).

The third, fourth and fifth members of the series (II, n = 2, 3 and 4) were isolated and characterised by the chromatographic identification of oligomers of the series II they furnished on partial hydrolysis and by the gas chromatographic identification of the methyl glycosides of 2,3,4,6-tetra- and 2,3,6-tri-O-methyl-D-galactose as methanolysis products of the methylated oligosaccharides. The ionophoretic mobilities of these oligosaccharides agreed favourably with the values quoted by Bouveng and Meier (83) for the same oligosaccharides isolated from the partial acid hydrolysate of the galactan from Norwegian spruce compression wood.

Chromatographic evidence indicated the presence in the last fraction from the charcoal-Celite column of a mixture of the fifth, sixth and seventh members of the series (II, n = 4, 5 and 6). The fraction yielded oligomers of the series II on partial hydrolysis and the methyl glycosides of 2,3,4,6-tetra- and 2,3,6-tri-O-methyl-D-galactose which were shown by gas chromatography to be methanolysis products of the methylated fraction.

In addition to the homologous series II of sugars a small amount of another homologous series appeared to be present as products of partial hydrolysis of the ME2 polysaccharide complex since many of the fractions stained yellow and red with p-anisidine and gave on hydrolysis small amounts of arabinose and glucose together with galactose. Although this oligosaccharide series could not be separated from the galactose-containing series II because of the similarity in chromatographic mobilities it seemed probable, by virtue of the red stain produced with p-anisidine, that the second oligosaccharide series had reducing end group units of arabinose.

Partial hydrolysis of the polysaccharide complex ME2 with N - sulphuric acid furnished a small quantity of insoluble degraded polysaccharide, a degraded polysaccharide precipitated from the hydrolysate by the addition of acetone, and a mixture of mono- and oligosaccharides. The insoluble degraded polysaccharide on hydrolysis yielded galacturonic acid, glucose, xylose and rhamnose and the precipitated degraded polysaccharide gave galacturonic acid with traces of xylose and rhamnose.

The oligosaccharide components were separated from the monosaccharides on charcoal and further separated into neutral and acidic fractions on diethylaminoethyl-Sephadex. The neutral fraction contained 2-O-methylxylose and 2-O-methylfucose and the galactose-containing oligosaccharides II isolated from the 0.5N-sulphuric acid partial hydrolysate. The acidic oligosaccharides were fractionated on a diethylaminoethyl-Sephadex column followed where necessary by partition chromatography on cellulose filter sheets.

The main component (O_1) was the aldobiouronic acid, 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose V which was characterised by the formation of the crystalline methyl glycoside penta methyl ether of the acid which was identical (m.p. and mixed m.p., specific rotation and X-ray powder photograph) with an authentic specimen.

α-D-Gal*p*A 1→2 L-Rha V

The second acidic oligosaccharide (O_2) was chromatographically and electrophoretically identical to 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose VI and the gas chromatographic identification of the methyl ester methyl glycoside of 2,3,4-tri-*O*-methyl-D-glucuronic acid and the methyl glycosides of 2,3,4-tri- and 2,3,5-tri-*O*-methyl-D-galactose as products of methanolysis of the methylated oligosaccharide, further substantiated the formulation VI.

β-D-G*p*A 1→6 D-Gal VI

The third acidic oligosaccharide (O_3) was an aldobiouronic acid containing glucuronic acid and fucose moieties. The methyl ester methyl glycoside of 2,3,4-tri-*O*-methyl-D-glucuronic acid was identified by gas chromatography as a product of methanolysis of the methylated oligosaccharide but other peaks indicated on the gas chromatogram could not be identified because of the lack of authentic dimethylfucose standards.

The periodate-pararosaniline spray reagent developed by Buchanan and Hardy (84) was employed to provide information concerning the point of attachment of the glucuronic acid unit to the fucose residue. The formation of a yellow colour on treatment of a carbohydrate with this reagent was an indication that a derivative of malonaldehyde had been produced by sodium periodate oxidation, whereas the formation of a blue colour was an indication that other aldehydes had been formed.

The glucuronic acid unit linked to carbon atom 2 or 3 of the fucose moiety would be the only combination to yield a derivative of malonaldehyde on oxidation with sodium metaperiodate and the derived glycitol would only give a malonaldehyde type of structure on periodate oxidation if the glucuronic acid unit was linked to carbon atom 3 of the fucitol moiety.

The disaccharide and the corresponding glycitol (borohydride reduction) formed blue colours when treated with the reagent and it seemed probable on this evidence that the acid moiety was linked to carbon atom 4 of the fucose residue. The absence of a (1⟶2)-linkage was verified by the intense red colour produced when the oligosaccharide was treated with 2,3,5-triphenyltetrazolium chloride reagent (21).

The molecular rotation, -231, of the aldobiouronic acid compared to the sum of the molecular rotations, -182, of D-glucuronic acid and L-fucose tended to favour a β-type of linkage in the structure VII, which was assigned to the acidic oligosaccharide (O_3).

β-D-GpA 1→4 L-Fuc VII

The fourth acidic oligosaccharide (O_4) was composed of glucuronic acid and galactose residues and the gas chromatographic identification of the methyl ester methyl glycoside of 2,3,4-tri-O-methyl-D-glucuronic acid and the methyl glycoside of 2,3,6-tri-O-methyl-D-galactose as products of methanolysis of the methylated oligosaccharide, together with the negative specific rotation (-28°) indicated that the aldobiouronic acid was 4-O-(β-D-glucopyranosyluronic acid)-D-galactose VIII.

β-D-GpA 1→4 D-Gal VIII.

The fifth acidic oligosaccharide (O_5) contained galacturonic acid and rhamnose in the estimated (37,39) proportion of 1:1 and after reduction the ratio was 1.7:1, which indicated that the oligosaccharide was a tetrasaccharide composed of two units of galacturonic acid and rhamnose residues. The tetrasaccharide on partial acid hydrolysis gave galacturonic acid, rhamnose and 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose and these reducing sugars together with rhamnitol and 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnitol as products of partial hydrolysis of the potassium borohydride reduced tetrasaccharide.

The cleavage products from the methylated tetrasaccharide were shown by gas chromatography to be the methyl esters methyl glycosides of 2,3-di- and 2,3,4-tri-*O*-methyl-*D*-galacturonic acid and the methyl glycoside of 3,4-di-*O*-methyl-*L*-rhamnose. Paper and gas chromatographic evidence indicated that the cleavage products of the reduced methylated oligosaccharide were 3,4-di-*O*-methyl-*L*-rhamnose and 2,3-di- and 2,3,4-tri-*O*-methyl-*D*-galactose. The methyl glycosides of 3,4-di-*O*-methyl-*L*-rhamnose and 2,3,4,6-tetra-, 2,3,6-tri-, and 2,3,4-tri-*O*-methyl-*D*-galactose were shown by gas chromatography, to be the products of methanolysis of the remethylated, reduced methylated oligosaccharide. The 2,3,4-tri-*O*-methyl-*D*-galactose probably arose from incomplete remethylation of the reduced methylated oligosaccharide. The molecular rotation, +688, of the oligosaccharide compared to the sum of the molecular rotations, +585, for two galacturonosylrhamnose V units tended to favour a β-type of linkage between these two constituent units in the structure IX which was assigned to the oligosaccharide.

α-*D*-Gal*p*A 1→2 β-*L*-Rha*p* 1→4 α-*D*-Gal*p*A 1→2 *L*-Rha IX

The sixth oligosaccharide fraction (O_6) contained galacturonic acid and rhamnose in the estimated (37,39) molar proportion of 1.8:1 and after reduction with potassium borohydride the ratio was 1.2:1. On partial acid hydrolysis the fraction yielded galacturonic acid, rhamnose and 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose and these sugars together with a trace of rhamnitol were obtained on partial acid hydrolysis of the derived glycitol (borohydride reduction). Gas chromatographic evidence indicated that the cleavage products from the methylated fraction were the methyl esters methyl glycosides of 2,3,4-tri- and 2,3-di-O-methyl-D-galacturonic acid with a small amount of the methyl glycoside of 3,4-di-O-methyl-L-rhamnose. The fraction was subsequently found to be heterogeneous containing the tetrasaccharide IX and another oligosaccharide with similar chromatographic mobility which on the evidence presented could not be assigned a structure.

The seventh oligosaccharide (O_7) contained galacturonic acid and rhamnose in the estimated (37,39) molar proportions of 1.2:1 and after reduction with potassium borohydride the ratio was 2.3:1. On partial acid hydrolysis, galacturonic acid, rhamnose and 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose were detected and these sugars together with rhamnitol were obtained on partial acid hydrolysis of the derived glycitol (borohydride reduction). The cleavage products from the methylated oligosaccharide were shown by gas chromatography to be the methyl ester methyl glycoside of 2,3,4-tri-O-methyl-D-galacturonic acid and the methyl glycoside of 3,4-di-O-methyl-L-rhamnose together with other compounds giving peaks on the chromatogram which could not be identified. The gas chromatogram of the products from the methanolysis of remethylated, reduced methylated oligosaccharide contained a complex mixture of peaks which could not be interpreted. On the evidence presented no structure was assigned to this oligosaccharide.

The eighth oligosaccharide (O_8) gave only galacturonic acid on hydrolysis and was chromatographically and electrophoretically indistinguishable from 4-O-α-D-galactopyranosyluronic acid-D-galactopyranosyluronic acid X. The sugar furnished a calcium salt with a specific rotation similar to calcium digalacturonate.

α-D-GalpA 1→4 D-GalpA X

The ninth oligosaccharide (O_9) gave only galacturonic acid on hydrolysis and was chromatographically and electrophoretically indistinguishable from O-α-D-galactopyranosyluronic acid-(1→4)-O-α-D-galactopyranosyluronic acid-(1→4)-D-galactopyranosyluronic acid XI. The sugar furnished a calcium salt with a specific rotation similar to calcium trigalacturonate.

α-D-GalpA 1→4 α-D-GalpA 1→4 D-GalpA XI

The information obtained from the partial depolymerisation experiments on the polysaccharide complex isolated from soybean meal is summarised in the following flowsheets.

DEPOLYMERISATION OF THE POLYSACCHARIDE COMPLEX FROM SOYBEAN MEAL.

Polysaccharide Complex

$\xrightarrow[\text{at } 100^\circ]{0.02\underline{\underline{N}}\text{-}H_2SO_4}$ Arabinose and fucose (later galactose and xylose)

$\xrightarrow[\text{deacetylation}]{\text{acetolysis}}$ Neutral oligosaccharides containing galactose, xylose, rhamnose and fucose + α-L-Fucp 1→2 D-Xyl XII

β-D-Galp 1→2 D Xyl XIII
(Chromatographic evidence only)

$\xrightarrow[\text{at } 100^\circ \text{ for 4 hr.}]{0.5\underline{\underline{N}}\text{-}H_2SO_4}$ Insoluble degraded polysaccharide $\xrightarrow{\text{Hydrolysis}}$ Galacturonic acid (+++), glucose (++) galactose (tr.) mannose (tr.), xylose (+++), rhamnose (+), methylated sugars (+).

+

Acetone precipitated degraded polysaccharide $\xrightarrow{\text{Hydrolysis}}$ Galacturonic acid (+++), galactose (tr.), xylose (+++), rhamnose (+), methylated sugars (+).

+

β-D-Galp-[(1→4)-β-D-Galp]$_n$-(1→4)-D-Galp II (n = 0→6)

DEPOLYMERISATION OF THE POLYSACCHARIDE COMPLEX FROM SOYBEAN MEAL.

Polysaccharide Complex

$\xrightarrow[\text{at } 100^\circ \text{ for 7 hr.}]{N\text{-}H_2SO_4}$ Insoluble degraded polysaccharide $\xrightarrow{\text{Hydrolysis}}$ Galacturonic acid (+++), glucose (+++), xylose (++), rhamnose (+).

\+

Acetone precipitated degraded polysaccharide $\xrightarrow{\text{Hydrolysis}}$ Galacturonic acid, xylose (tr.), rhamnose (tr.).

\+

α-D-GalpA 1→2 L-Rha V

β-D-GpA 1→6 D-Gal VI

β-D-GpA 1→4 L Fuc VII

β-D-GpA 1→4 D-Gal VIII

α-D-GalpA 1→4 D-GalpA X

α-D-GalpA 1→4 α-D-GalpA 1→4 D-GalpA XI

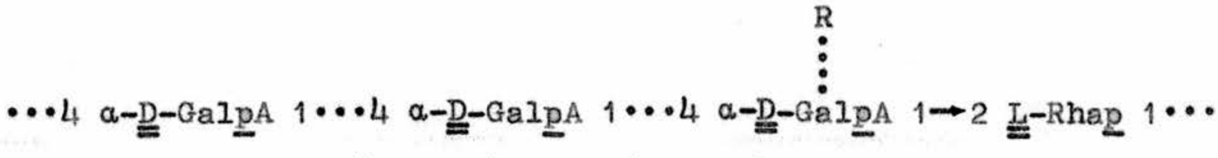
α-D-GalpA 1→2 β-L-Rhap 1→4 α-D-GalpA 1→2 L-Rha IX

The isolation from partial depolymerisation of the soybean meal polysaccharide complex of a degraded polysaccharide largely composed of galacturonic acid residues, together with di- and trigalacturonic acids X and XI, suggested that the polysaccharide complex could be classified as a pectic substance. The terms pectic substances or pectic materials have been used as a group designation for complex colloidal carbohydrates which contain galacturonic acid residues existing in linear chain-like combination (85).

Although pectic acids have been shown to be polymers of (1→4)-linked α-D-galacturonic acid residues XIV (86) more recent studies of pectic acids and pectins have suggested that neutral sugars exist as integral constituents of these polysaccharides XV.

$$\cdots 4\ \alpha\text{-}\underline{\underline{\mathrm{D}}}\text{-Gal}\underline{p}\text{A}\ 1\rightarrow 4\ \alpha\text{-}\underline{\underline{\mathrm{D}}}\text{-Gal}\underline{p}\text{A}\ 1\rightarrow 4\ \alpha\text{-}\underline{\underline{\mathrm{D}}}\text{-Gal}\underline{p}\text{A}\ 1\rightarrow 4\ \alpha\text{-}\underline{\underline{\mathrm{D}}}\text{-Gal}\underline{p}\text{A}\ 1\cdots$$

XIV



$$\cdots 4\ \alpha\text{-}\underline{\underline{\mathrm{D}}}\text{-Gal}\underline{p}\text{A}\ 1\cdots 4\ \alpha\text{-}\underline{\underline{\mathrm{D}}}\text{-Gal}\underline{p}\text{A}\ 1\cdots 4\ \alpha\text{-}\underline{\underline{\mathrm{D}}}\text{-}\overset{\overset{\displaystyle \mathrm{R}}{\vdots}}{\text{Gal}\underline{p}\text{A}}\ 1\rightarrow 2\ \underline{\underline{\mathrm{L}}}\text{-Rha}\underline{p}\ 1\cdots$$

XV ; $R = (\underline{\underline{\mathrm{L}}}\text{-Ara}\underline{f})_n$ or $(\underline{\underline{\mathrm{D}}}\text{-Gal}\underline{p})_n$

The pectic substance from sisal (87), which resisted attempted fractionation, contained rhamnose, arabinose, and galactose together with galacturonic acid as the main constituent sugar. The fractionated pectic acids from several fruits and vegetables (88) yielded on hydrolysis galacturonic acid, galactose, arabinose, xylose and rhamnose and these sugars were also constituents of the pectic complex isolated from fresh-water green algae Nitella translucens (89). Sugar beet pectic acid was fractionated on diethylaminoethylcellulose and the fractions isolated from the column contained arabinose and galactose together with galacturonic acid (90,29).

Soybean hulls on extraction with cold and hot water yielded pectic materials together with galactomannans I and II. These pectic acids were found to contain significant amounts of neutral sugars. Subsequent extraction of the hulls with 0.5% aqueous ammonium oxalate yielded more pectic material which was examined in greater detail (91). Chromatography of the pectic acid on diethylaminoethylcellulose showed that the major portion of the polysaccharide was eluted in a single band and hydrolysis of this component gave galactose, arabinose, xylose, fucose, rhamnose and traces of 2-O-methylxylose and 2-O-methylfucose, the sugars also present in the parent pectic acid. Partial acid hydrolysis of the pectic acid furnished a complex mixture of acidic oligosaccharides from which 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose V was isolated and characterised ; preliminary methylation results indicated the presence in the structure of (1→4)-linked D-galactose units.

Recently in this laboratory (92) commercial citrus pectin was fractionated into a neutral galactan and an acidic component, which being devoid of galactan contaminant, afforded on partial hydrolyses, 4-O-β-D-galactopyranosyl-D-galactose and the corresponding trisaccharide, together with oligomers of galacturonic acid and 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose V. In this respect citrus pectic acid is similar to the polysaccharide complex from soybean cotyledon meal.

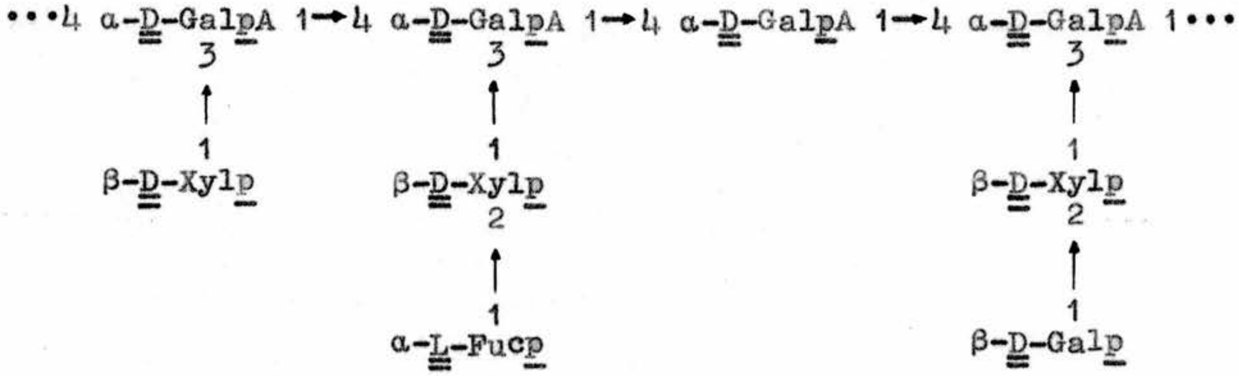
The isolation of the homologous series II of galactose-containing oligosaccharides from the partial acid hydrolysate of the soybean polysaccharide complex indicated that long side chains of polygalactose units (XVI) were present in the structure. Further proof of their existence in the polysaccharide complex was provided by the gas chromatographic identification of a very substantial amount of the methyl glycoside of 2,3,6-tri-O-methyl-D-galactose as a product of methanolysis of the methylated polysaccharide complex (93).

•••4 β-D-Gal*p* 1→4 β-D-Gal*p* 1→4 β-D-Gal*p* 1→4 β-D-Gal*p* 1•••

XVI

This structural feature XVI has been found in the galactans associated with pectic substances (94,95) and those isolated from *Strychnos nux-vomica* seeds (96) and Norwegian spruce compression wood (83).

The rapid release of arabinose units on mild hydrolysis of the soybean meal pectic complex suggested that these sugar residues were present in the furanose form, possibly mutually linked and probably occupying peripheral sites in the molecular structure; fucose was also released rapidly and it has been observed previously that L-fucopyranosyl residues are readily released from terminal positions in tragacanthic acid, the acid component of gum tragacanth (97,98). The isolation from a more drastic partial hydrolysis of a degraded polysaccharide composed largely of galacturonic acid and xylose residues, together with the detection of 2-O-α-L-fucopyranosyl-D-xylose XII and 2-O-β-D-galactopyranosyl-D-xylose XIII as deacetylation products from graded acetolysis of the soybean polysaccharide complex, provided a further analogy with the polysaccharide, tragacanthic acid XVII (98).

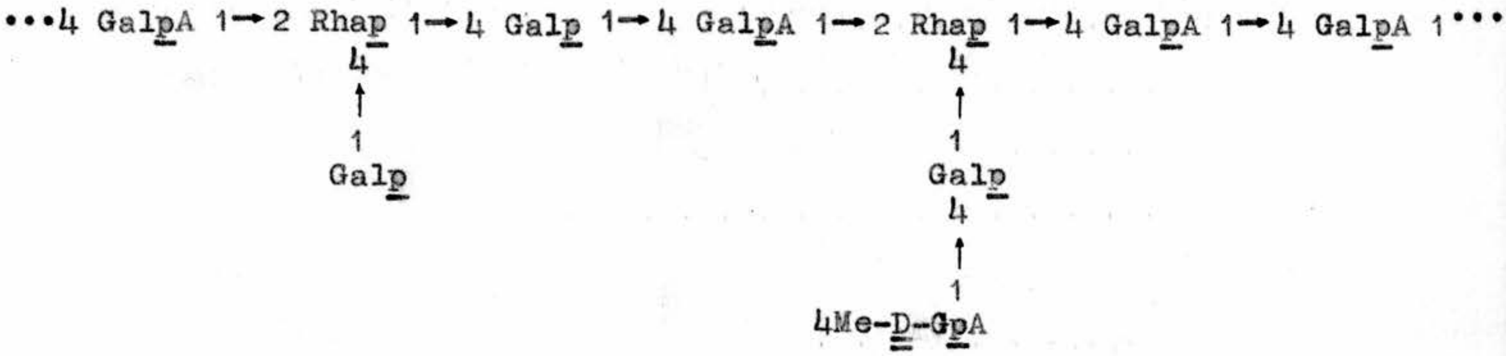


XVII

The identification by gas chromatography of the methyl glycosides of 2,3,4-tri-O-methyl-L-fucose, 2,3,4,6-tetra-O-methyl-D-galactose and 3,4-di- and 2,3,4-tri-O-methyl-D-xylose from the methanolysed methylated polysaccharide complex from soybean meal (93) provided additional support to the suggestion that the polysaccharide complex contained fucose, galactose and xylose residues linked in a similar manner to those in tragacanthic acid XVII.

The soybean polysaccharide complex, unlike tragacanthic acid, afforded the aldobiouronic acid 2-*O*-(α-D-galactopyranosyluronic acid)-L-rhamnose V and in this respect resembles the pectic acids isolated from lucerne (99), soybean hulls (91), citrus pectin (92) and grape juice (100), and in this respect also resembles the gum *Cochlospermum gossypium* (101, 102) and the gums of the *Khaya* and *Sterculia* genera (103-109). The isolation of this aldobiouronic acid V and the tetrasaccharide *O*-α-D-galactopyranosyluronic acid-(1⟶2)-*O*-β-L-rhamnopyranosyl-(1⟶4)-*O*-α-D-galactopyranosyluronic acid-(1⟶2)-L-rhamnose IX indicated that the polysaccharide complex contained chains of contiguous galacturonic acid and rhamnose residues, but it is not yet known here or in the case of pectic acids whether these oligosaccharides arise from L-rhamnose residues interposed at intervals between blocks of (1⟶4)-linked α-D-galacturonic acid residues as in the plant gums of the *Khaya* genus (103, 104) or whether they arise from an entirely separate portion of the structure.

Partial hydrolysis of the *K. grandifolia* gum (104) afforded the aldobiouronic acid 2-*O*-(α-D-galactopyranosyluronic acid)-L-rhamnose V together with 4-*O*-(4-*O*-methyl-D-glucopyranosyluronic acid)-D-galactose and *O*-D-galactopyranosyluronic acid-(1⟶2)-*O*-L-rhamnopyranose-(1⟶4)-D-galactose. The isolation of these oligosaccharides together with methylation results (104) led to the formulation of the following partial structure XVIII which incorporated the known structural features of the polysaccharide which were similar to those of the major polysaccharide component of *K. senegalensis* gum (105).



$$\begin{array}{ccccc}
\cdots 4\ \mathrm{Gal}\underline{p}\mathrm{A}\ 1\rightarrow 2 & \mathrm{Rha}\underline{p} & 1\rightarrow 4\ \mathrm{Gal}\underline{p}\ 1\rightarrow 4\ \mathrm{Gal}\underline{p}\mathrm{A}\ 1\rightarrow 2 & \mathrm{Rha}\underline{p} & 1\rightarrow 4\ \mathrm{Gal}\underline{p}\mathrm{A}\ 1\rightarrow 4\ \mathrm{Gal}\underline{p}\mathrm{A}\ 1\cdots \\
 & 4 & & 4 & \\
 & \uparrow & & \uparrow & \\
 & 1 & & 1 & \\
 & \mathrm{Gal}\underline{p} & & \mathrm{Gal}\underline{p} & \\
 & & & 4 & \\
 & & & \uparrow & \\
 & & & 1 & \\
 & & & 4\mathrm{Me}\text{-}\underline{\underline{\mathrm{D}}}\text{-}\mathrm{G}\underline{p}\mathrm{A} &
\end{array}$$

XVIII

Recently in this laboratory (106) the gums, *Sterculia setigera* (107), *S. urens* (108), *S. caudata* (109) and *Cochlospermum gossypium* (101), have furnished on partial acid hydrolysis the aldobiouronic acid 2-*O*-(α-D-galactopyranosyluronic acid)-L-rhamnose V and the aldotriouronic acids *O*-β-D-glucopyranosyluronic acid-(1→3)-*O*-α-D-galactopyranosyluronic acid -(1→2)-L-rhamnose and *O*-α-D-galactopyranosyluronic acid-(1→4)-*O*-α-D-galactopyranosyluronic acid-(1→2)-L-rhamnose together with other acidic oligosaccharides which suggested that, like the gums of the *Khaya* genus, the rhamnose residues in these gums were interposed between blocks of (1→4)-linked α-D-galacturonic acid residues and constituted points of branching in the main chain, but unlike the *Khaya* gums in that galacturonic acid residues also formed points of branching in the main chain.

The isolation of 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose VI, 4-*O*-(β-D-glucopyranosyluronic acid)-D-galactose VIII and 4-*O*-(β-D-glucopyranosyluronic acid)-L-fucose VII from the partial hydrolysate of the soybean meal polysaccharide complex suggested that glucuronic acid residues may be present in the structure as end groups terminating side chains containing galactose and fucose residues. Glucuronic acid has been normally found in the gums of the *Acacia* genus occupying terminal positions in the side chains of (1→6)-linked β-D-galactose residues (110).

Methylation studies on the soybean meal polysaccharide complex and studies on the partially degraded polysaccharide will have to be completed before any definite structure can be formulated incorporating the structural features already elucidated. It can be stated however, that the soybean cotyledon meal polysaccharide complex contains a number of acidic polysaccharide components of apparently similar type which appear to have certain structural features in common with pectic acids, tragacanthic acid and certain plant gums, particularly those of the *Khaya* and *Sterculia* genera.

SECTION II

EXPERIMENTAL

EXTRACTION SCHEME A.

Dehulled and defatted meal (800 g.) was successively extracted with cold and hot water. The residual material from these extracts underwent the following extraction procedures.

(i) The residue was extracted five times with 0.5% aqueous ammonium oxalate for 4 hr., at 80° and the resulting solutions were centrifuged. The combined supernatant liquid was concentrated to half its original volume and acidified to pH 4.5 with acetic acid in an attempt to precipitate protein. The insoluble material (E1), (47 g.), was removed at the centrifuge and the polysaccharide material (E2),(30 g.), precipitated with ethanol. The precipitate was separated, washed successively with ethanol, acetone and ether, and dried in a desiccator.

(ii) Ammonium oxalate extracted material was extracted five times with 0.2% aqueous sodium hydroxide for 24 hr., at room temperature. The extracts were centrifuged, the supernatant liquid was acidified to pH 4.5 and the proteinaceous material (E3), (270 g.), was removed at the centrifuge. By the addition of ethanol to the concentrated supernatant liquid a polysaccharide fraction (E4), (5 g.), was precipitated, separated and dried by solvent exchange.

(iii) The residue from procedure (ii) was five times extracted with 5% aqueous sodium hydroxide in the manner described above to yield a protein fraction (E5), (111 g.), and a polysaccharide fraction (E6), (25 g.). The residue (E7), (30 g.) from the above procedure was washed free from alkali and dried by solvent exchange.

The following Table I indicates the protein content (% N x 6.25) of fractions E1 - E7 and the relative proportions of sugars given on total hydrolysis.

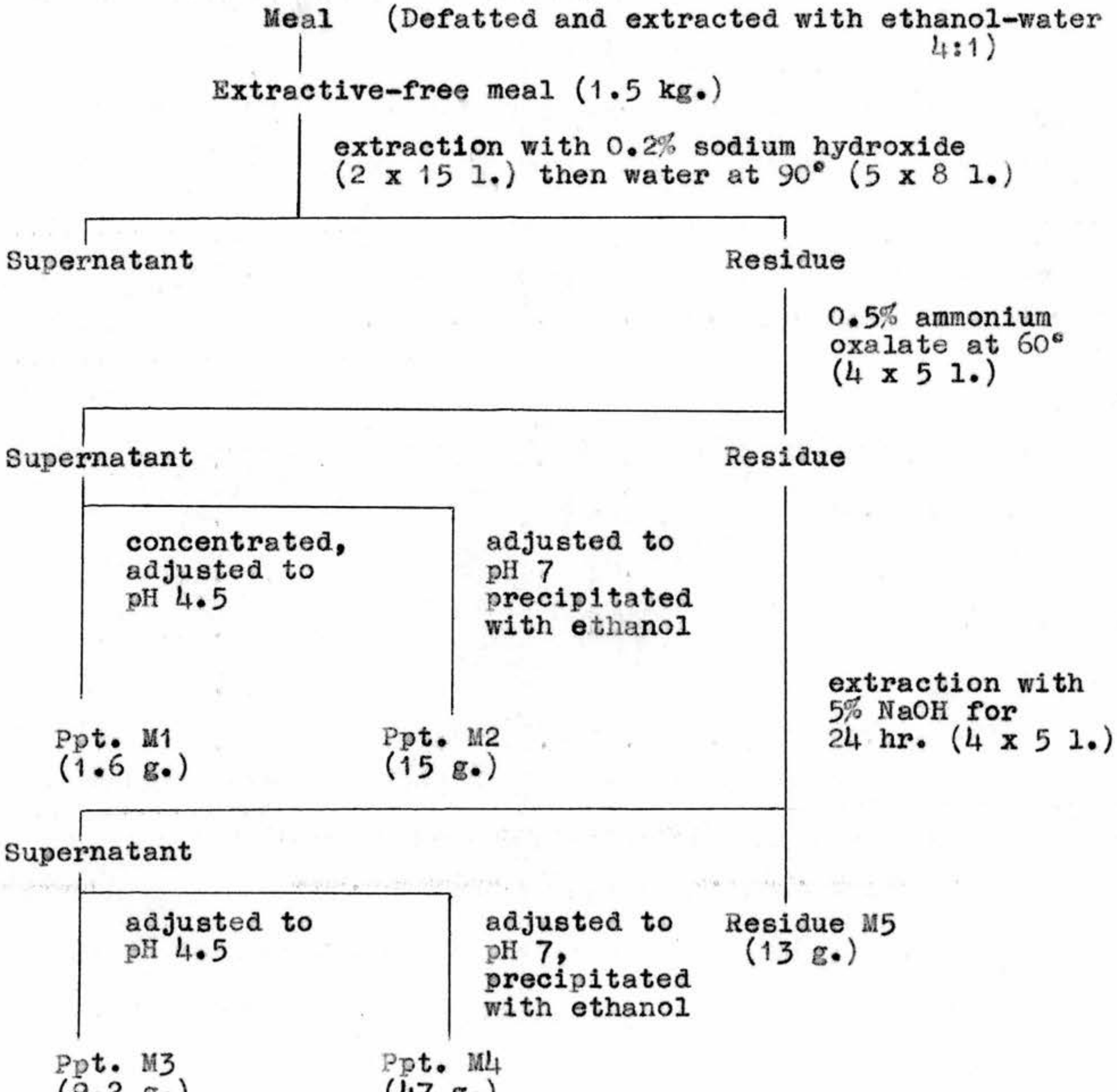
TABLE I.

Fraction	Protein %	Component Sugars									
		GalA.	Gal.	Man.	Glu.	Ara.	Xyl.	Fuc.	Rib.	Rha.	MeS.
E1	16.9	++	+++	++	++	+++	++	+	+	+	-
E2	9.4	++	+++	tr.	tr.	+++	++	+	+	++	+
E3	79.6	++	++	+	+	++	++	tr.	+	+	-
E4	45.8	++	+++	tr.	tr.	++	++	+	+	+	+
E5	57.1	++	++	+	+	+	++	+	-	+	-
E6	5.09	++	++	+	+	+	++	+	+	++	+
E7	15.3	++	+	tr.	tr.	tr.	++	tr.	-	++	-

MeS. = methylated sugars. tr. = trace.

EXTRACTION SCHEME B.

The extraction scheme is summarised in the following flow diagram.



This scheme involved an extraction of the meal with cold very dilute alkali to solubilise the protein present prior to ammonium oxalate and sodium hydroxide extraction procedures otherwise the extraction techniques were the same as those indicated in Scheme A.

The following Table 2 indicates the protein content of fractions M1 - M5 and the relative proportions of sugars given on hydrolysis.

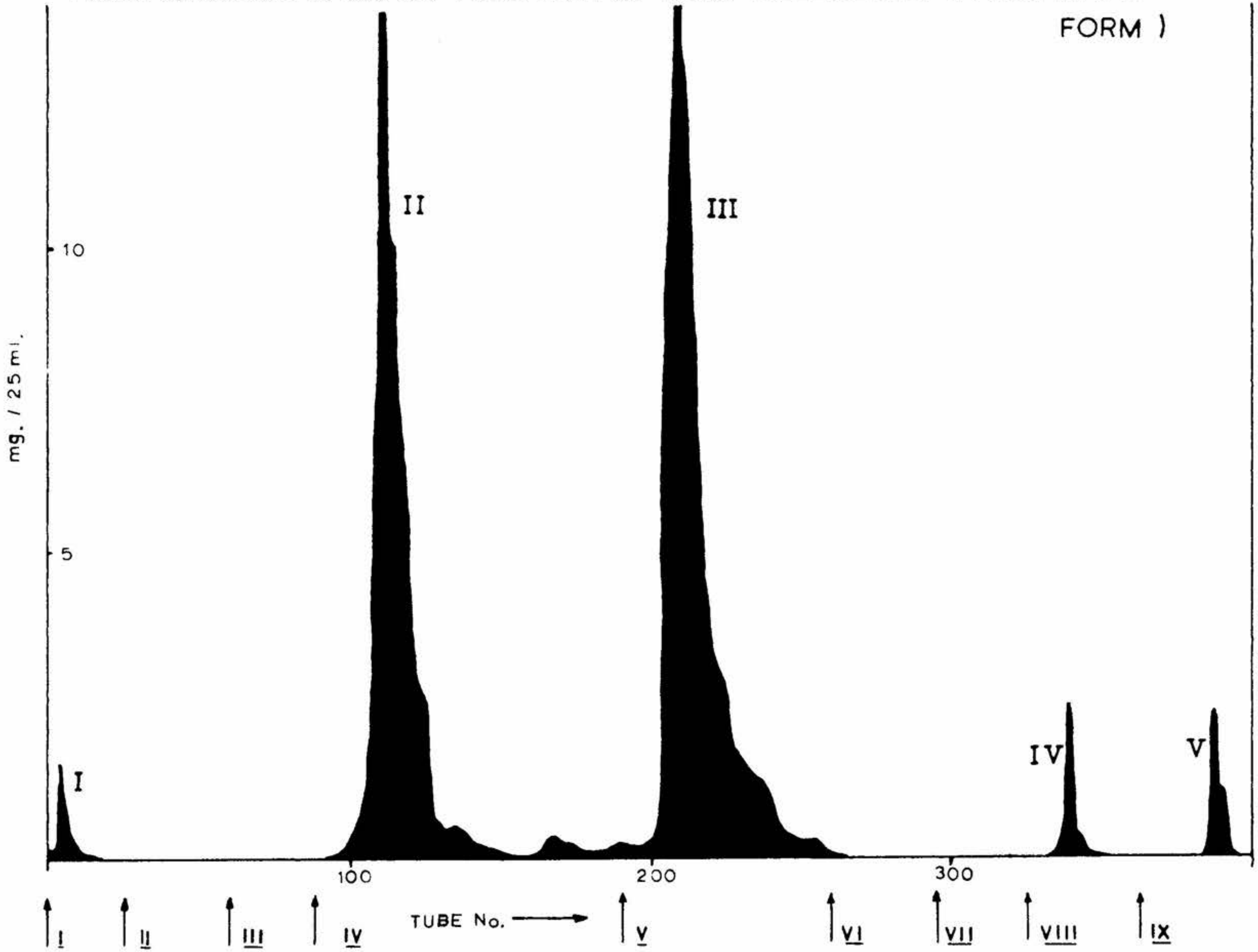
TABLE 2.

Fraction	Protein %	Component Sugars									
		GalA.	Gal.	Man.	Glu.	Ara.	Xyl.	Fuc.	Rib.	Rha.	MeS.
M1	52.4	++	++	++	++	++	++	+	+	+	tr.
M2	3.7	++	++	tr.	tr.	++	++	++	+	++	+
M3	40.0	++	++	+	+	++	++	+	tr.	+	tr.
M4	2.3	++	++	++	++	++	++	++	+	++	+
M5	10.6	+	+	tr.	++	+	++	-	-	+	-

MeS. = methylated sugar tr. = trace

The soybean meal 0.5% **aqueous** ammonium oxalate extract M2 had $[\alpha]_D$ +54° (*c* 1.0 in water), U.A. 27.0% (uronic anhydride determined by decarboxylation), and ash content 0.85%.

FRACTIONATION of the M2 FRACTION on DEAE-CELLULOSE (PHOSPHATE FORM)



Eluate : I 0·025M , II 0·05 M, III 0·1 M, IV 0·25M, V 0·5M-PHOSPHATE BUFFER.

VI 0·1 M, VII 0·25 M, VIII 0·5 M-NaCl. IX 0·2 N-NaOH.

Fractionation of Soybean Meal Polysaccharide Extract M2 on Diethylaminoethylcellulose.

(1) A sample (450 mg.) of the polysaccharide mixture was dissolved in water (20 ml.) and added to a column (30 g.) of diethylaminoethylcellulose in the phosphate form. The column was eluted successively with 0.025, 0.05, 0.1, 0.25 and 0.5 M-sodium dihydrogen phosphate buffer at pH 6.1, and then with 0.1, 0.25 and 0.5 M-sodium chloride solution and finally with 0.2 N-aqueous sodium hydroxide. Fractions (25 ml.) were collected and analysed by the phenol-sulphuric acid method and a plot of sugar content per 25 ml. fractions against tube number is shown on the accompanying diagram. Five polysaccharide fractions were detected and the contents of the appropriate tubes were combined, dialysed, concentrated and freeze-dried.

The results of examination of these fractions are indicated in the following Table 3.

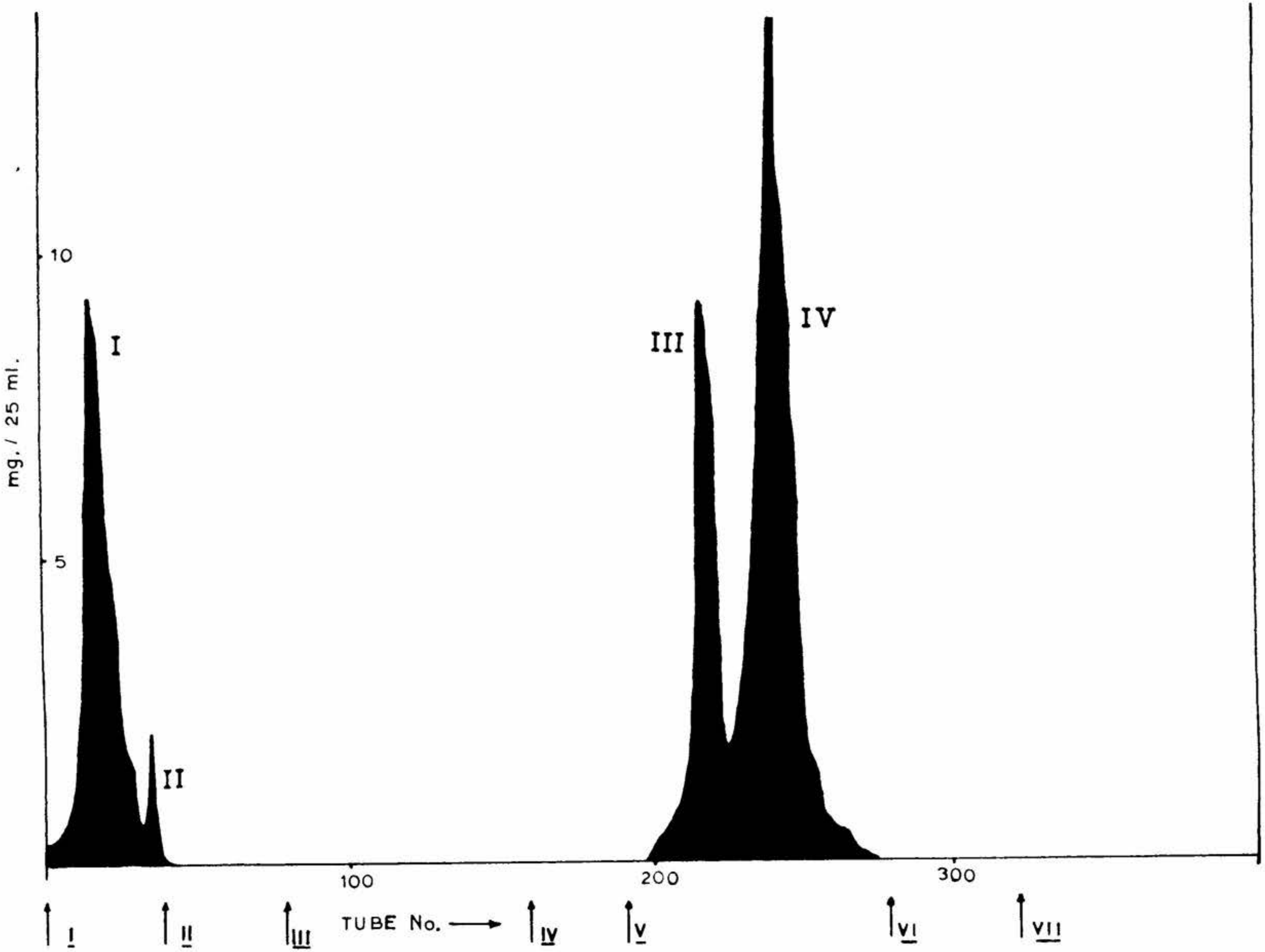
TABLE 3.

Fract-ion	Wt. (mg.)	$[\alpha]_D$	U.A.	Component Sugars								
				GalA.	Gal.	Man.	Glu.	Ara.	Xyl.	Fuc.	Rha.	MeS.
1	0.5	-	-	tr.	+	+	+	+	+	-	-	-
2	88	+40°	30%	+	++	-	-	++	++	+	+	+
3	191	+56°	32%	+	+	-	-	++	+++	+	+	+
4	7	-	-	+++	++	+	+	+++	+++	+	+	-
5	7	-	-	+++	++	++	++	+++	+++	+	+	-

MeS. = Methylated sugars

U.A. = Uronic anhydride determined by decarboxylation.

FRACTIONATION of the M2 FRACTION on DEAE-CELLULOSE (ACETATE FORM)



Eluate: I 0·025 M, II 0·04 M, III 0·1 M, IV 0·2 M-ACETATE BUFFER

V 0·2 M, VI 0·5 M, VII 1·0 M-NaCl in 0·02 M-ACETATE BUFFER

(ii) A sample of the polysaccharide mixture M2 was dissolved in water (20 ml.) and absorbed on a column (30 g.) of diethylaminoethylcellulose in the acetate form. The column was eluted with 0.025, 0.04, 0.1 and 0.2 M-sodium acetate buffer at pH 4.05 and then with 0.2, 0.5 and 1.0 M-sodium chloride in 0.02 M-sodium acetate buffer at pH 4.05. Column fractions (25 ml.) were collected and analysed by the phenol-sulphuric acid method. A plot of sugar content per 25 ml. fractions against tube number, as shown opposite, indicated the presence of three major and a very minor fraction, all of which were isolated as previously described.

Examination of these fractions gave the data shown in the following Table 4.

TABLE 4.

Fraction	Wt. (mg.)	$[\alpha]_D$	U.A.	Component Sugars								
				GalA.	Gal.	Man.	Glu.	Ara.	Xyl.	Fuc.	Rha.	MeS.
1	113	+42°	21%	++	++++	tr.	tr.	++	+++	++	+++	+
2	3	-	-	tr.	-	++	+	++	-	-	-	-
3	66	+23°	27%	++	++	tr.	-	+++	+++	++	++	+
4	195	+77°	41%	+++	++	tr.	-	+++	++++	++	++	+

MeS. = Methylated sugars.
U.A. = Uronic anhydride, determined by decarboxylation.
tr. = trace.

Attempted Fractionation of the Ammonium Oxalate Extract M2 by Precipitation Techniques.

a) Salt formation with cupric acetate.

Aqueous cupric acetate (7%) was added to a 1% solution of the polysaccharide mixture in water to form a precipitate. The cupric acetate solution was added until complete precipitation had occurred and the insoluble material was removed at the centrifuge. Ethanol (5 vol.) was added to the supernatant but no further precipitation occurred. The polysaccharide mixture appeared to be completely precipitated as insoluble copper salts and it appeared unlikely that fractionation could be achieved by this method.

b) Salt formation with calcium chloride.

Aqueous calcium chloride solution (10%) was added to a 1% solution of the polysaccharide in water but no precipitate was formed. Ethanol (1 vol.) was added to the mixture and a precipitate separated and was removed at the centrifuge. The addition of ethanol (5 vol.) to the supernatant failed to induce further precipitation. It seemed unlikely that the fractionation of the mixture could be obtained by variations in the solubility characteristics of the calcium salts.

c) Salt formation with cetavlon (cetyl-trimethyl-ammonium bromide).

Aqueous cetavlon solution (2%) was added to a 1% aqueous solution of the polysaccharide mixture and an immediate precipitate formed. Precipitant was added until complete precipitation had occurred. The insoluble material was removed at the centrifuge and the centrifugate adjusted to pH 10.0 with ammonium hydroxide in an attempt to precipitate additional material. No precipitate formed and the addition of alcohol (2 vol.) also failed to promote precipitation. The polysaccharide mixture again appeared to precipitate uniformly.

Small-scale Partial Hydrolyses of Soybean Meal Polysaccharide Fraction M2.

(1) With 0.02N-sulphuric acid.

The polysaccharide (0.25 g.) was heated in 0.02 N-sulphuric acid (12.5 ml.) on a water bath at 100°. Samples (2 ml.) were withdrawn at intervals, neutralised with barium carbonate, treated with Amberlite IR-120(H) resin, were concentrated, and examined by chromatography in solvents A and D. The results indicated the release after 1 hr. of small amounts of arabinose and fucose, after 2 hr. these sugars together with traces of galactose, after 3 - 6 hr. greatly increased amount of arabinose relative to fucose and galactose, and after 6 - 11 hr. traces of xylose in addition to the other sugars already mentioned.

(2) With N-sulphuric acid.

A sample of the polysaccharide (0.5 g.) was dissolved in water (12.5 ml.) and 2 N-sulphuric acid (12.5 ml.) was added. The solution was heated on a boiling water bath and samples (1 ml.) were taken hourly from the commencement of the experiment, neutralised with barium carbonate, were filtered and deionised with Amberlite IR-120(H) resin, concentrated, and were examined by chromatography in solvents A and D. The results indicated the maximum production of acidic oligosaccharides after 4 - 8 hr. During the early stages of the hydrolysis a neutral disaccharide, $R_{glucose}$ 0.3 and 0.4 in solvents A and D, was detected. This oligosaccharide was later shown to be galactobiose.

A further quantity (0.5 g.)of the polysaccharide was heated in N-sulphuric acid for 4 hr. at 100°. A flocculent residue which remained after this treatment was removed at the centrifuge and degraded polysaccharide was precipitated by the addition of acetone (1 vol.); acetone was removed by evaporation and the acidic solution was neutralised by shaking with methyldi-n-octylamine (5% v/v in chloroform) and concentrated to a syrup. The syrup in water (5 ml.) was added to a column of diethylaminoethyl-Sephadex (2 g. formate form).

The column was eluted with water to yield a mixture of neutral sugars, including galactose, mannose, glucose, arabinose, xylose, fucose, rhamnose and methylated sugars. This mixture was reduced with potassium borohydride and hydrolysed with 0.1 N-sulphuric acid for 4 hr., neutralised with barium carbonate, deionised with Amberlite IR-120(H) resin, concentrated and examined by chromatography in solvents A and D. Only trace quantities of the monosaccharides were detected indicating that very little, if any, neutral oligosaccharides were present in this fraction.

Elution of the column with a gradient 0 - 0.5 N-formic acid gave five main fractions. Fraction 1 contained an acidic oligosaccharide, R_{GalA} 0.80 in solvent A, which gave galacturonic acid and rhamnose on hydrolysis; fraction 2 contained galacturonic acid and two oligosaccharides R_{GalA} 0.80 and 0.57 in solvent A; fraction 3 contained a single oligosaccharide R_{GalA} 0.11, which gave galacturonic acid and rhamnose on hydrolysis; fraction 4 and 5 each contained a single component, R_{GalA} 0.22 and 0.05, probably di- and trigalacturonic acid respectively.

(3) Acetolysis Followed by Deacetylation.

The polysaccharide (1 g.) was dispersed in acetic anhydride (25 ml.) and acetic acid (25 ml.) and concentrated sulphuric acid (2.5 ml.) was added dropwise to the cooled solution. The acetolysis mixture was shaken vigorously for 1 hr. after which time the polysaccharide had completely dissolved. The solution was kept at room temperature and samples (10 ml.) were withdrawn every 24 hr., poured into ice-water (50 ml.), and the solutions were brought to pH 4-5 by the addition of sodium hydrogen carbonate. Acetylated sugars were extracted with chloroform, and the solutions dried over anhydrous sodium sulphate and concentrated. The resulting syrups were dissolved in methanol (20 ml.) and chloroform (5 ml.), 0.5 N-barium methoxide (5 ml.) was added, and the solutions were allowed to stand for 24 hr. at 0°. Dilute sulphuric acid was added to pH 7, barium sulphate was removed by centrifugation, and the solutions were concentrated and examined by chromatography in solvents A, B and D. The results indicated that the optimum time for the formation of oligosaccharides was after acetolysis for 72 hr. The various mixtures, which contained qualitatively similar mixtures of monosaccharides (galactose, mannose, arabinose, xylose, fucose and rhamnose and traces of methylated sugars) and oligosaccharides, were absorbed on a squat charcoal column (10 g.) and eluted with water to remove monosaccharides. Oligosaccharides were eluted with 35% aqueous ethanol and chromatographed in solvents A and D to indicate five main bands. The five oligosaccharide fractions were separated by chromatography on filter sheets, and hydrolysis of each indicated the main constituent sugars as shown in the following Table 5.

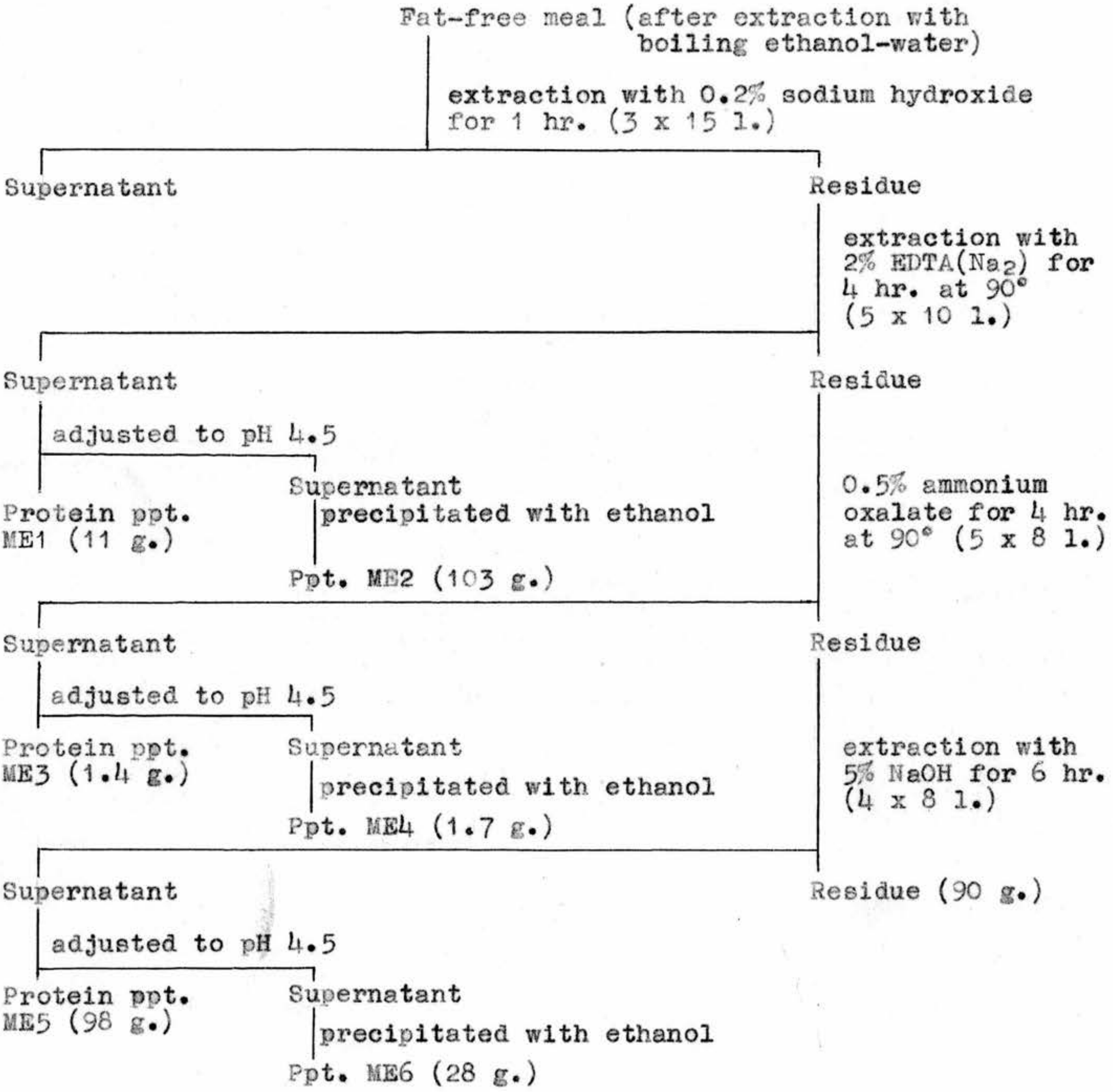
TABLE 5.

Oligosaccharide Fraction	Chromatographic Mobilities $R_{glucose}$		Hydrolysis products
	Solvent A	Solvent D	
A	0.1	0.25	Gal (+++), Xyl (+++), Glu (+), Fuc (+), Rha (+), Ara (tr.)
B	0.3	0.41	Gal (++), Xyl (++), Ara (tr.)
C	0.57	0.78	Gal (++++), Xyl (++++), Rha (++), Ara (+)
D	0.94	1.08	Gal (++), Ara (++), Xyl (++++), Rha (++),
E	1.10	1.22	Gal (++), Xyl (++++), Fuc (++)

The figures in parenthesis indicate approximate proportions of the hydrolysis products. Oligosaccharides C and E had similar chromatographic mobilities to those of 2-*O*-β-D-galactopyranosyl-D-xylose and 2-*O*-α-L-fucopyranosyl-D-xylose respectively.

EXTRACTION SCHEME C.

The extraction scheme is summarised in the following flow sheet.



The following Table 6 indicates the protein content (% N x 6.25) of fractions ME1 - ME6 and the relative proportions of sugars given on hydrolysis of the polysaccharide fractions.

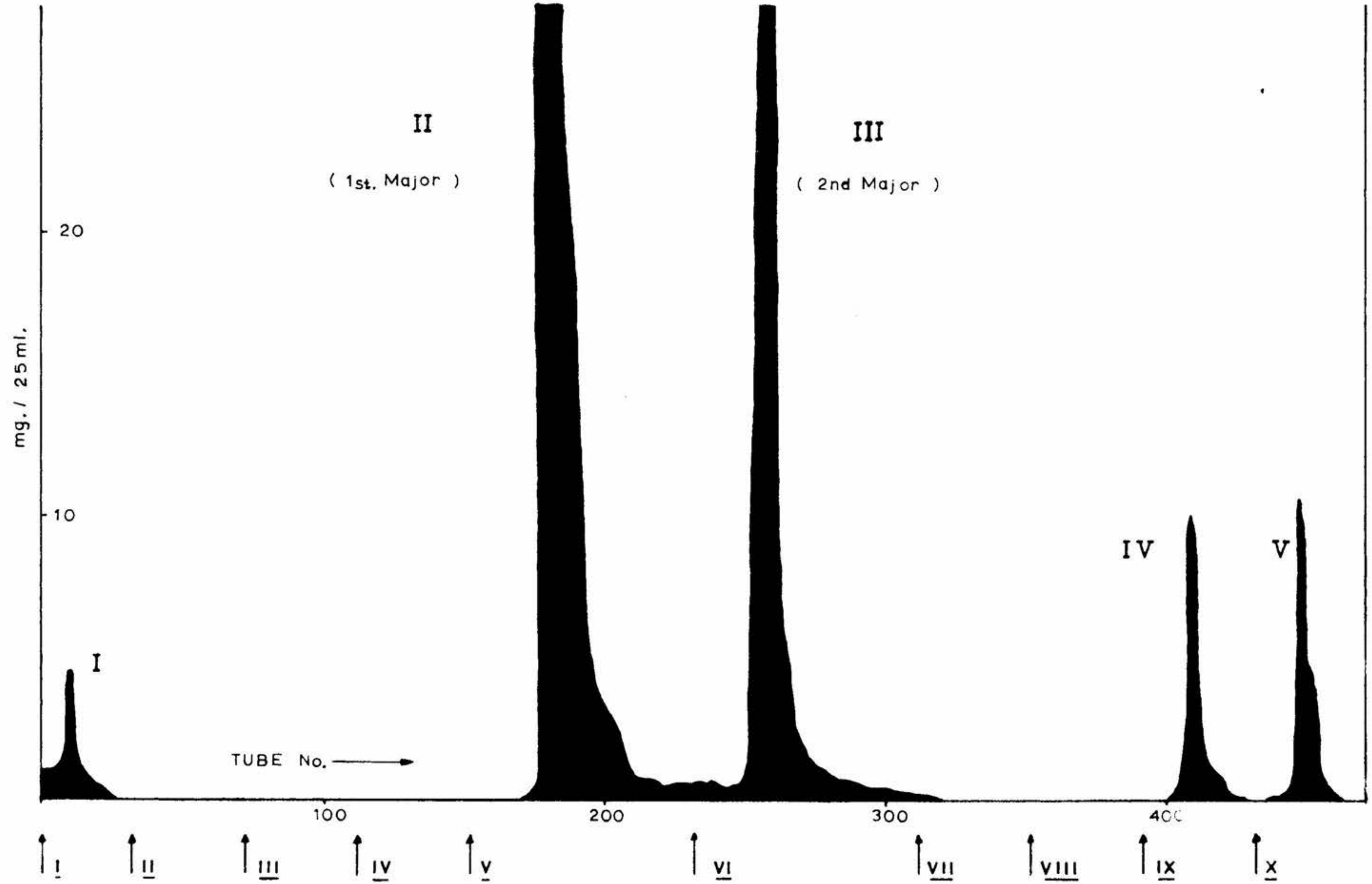
TABLE 6.

Fraction	Protein %	Component Sugars								
		GalA.	Gal.	Man.	Glu.	Ara.	Xyl.	Fuc.	Rha.	MeS.
ME1	55.3									
ME2	6.0	++	++	+	+	++	++	+	+	+
ME3	72.5									
ME4	6.8	++	++	+	+	++	++	+	+	+
ME5	80.0									
ME6	2.3	+++	++	+	+	++	++	+	+	+

MeS. = methylated sugar.

The soybean meal ethylenediaminetetraacetic acid disodium salt extract ME2 had $[\alpha]_D$ + 41°, (c 1.8 in water), U.A. 26% (uronic acid content determined by decarboxylation) and ash content 0.17%.

FRACTIONATION of the ME 2 FRACTION on DEAE-CELLULOSE (PHOSPHATE FORM)



Eluate : I Water. II 0·025 M , III 0·05 M , IV 0·1 M , V 0·25 M , VI 0·5 M - PHOSPHATE BUFFER .

VII 0·1 M , VIII 0·25 M , IX 0·5 M - NaCl .   X 0·2 N - NaOH .

Fractionation of Soybean Meal Polysaccharide Extract ME2 on Diethylaminoethylcellulose.

A sample (1 g.) of the polysaccharide mixture was dissolved in water (30 ml.) and added to a column (60 g.) of diethylaminoethylcellulose in the phosphate form. The column was eluted with water, 0.025, 0.05, 0.1, 0.25 and 0.5 M-sodium dihydrogen phosphate buffer at pH 6.1, and then with 0.1, 0.25 and 0.5 M-sodium chloride solution and finally with 0.2 N-aqueous sodium hydroxide. Fractions (25 ml.) were collected and analysed by the phenol-sulphuric acid method and a plot of sugar content per 25 ml. fractions against tube number is shown on the accompanying diagram. Five polysaccharide fractions were detected and the contents of the appropriate tubes were combined, dialysed, concentrated and freeze dried.

Samples (5 - 10 mg.) of the five fractions were heated in 0.5 N-sulphuric acid for 4 hr. at 100°. The resulting acid solutions were neutralised by shaking with Amberlite LA-2 resin (5% v/v in light petroleum) and were passed through columns of Amberlite IR-120(H) resin and concentrated to small volumes. Diethylaminoethyl-Sephadex (formate form) was added and the mixtures allowed to stand overnight. The Sephadex was removed by filtration and the filtrates concentrated to give mixtures of neutral sugars which were chromatographed in solvents A and B. The Sephadex was washed, on the filter, with 0.5 N-formic acid and the filtrates concentrated to yield mixtures of acidic sugars which were examined by chromatography in solvent A.

The major fractions 2 and 3 were subjected to graded acetolysis. The fractions (50 mg.) were added to cold acetolysis mixtures [acetic anhydride (5 ml.), acetic acid (5 ml.), and concentrated sulphuric acid (0.5 ml.)] and the resulting solutions kept at room temperature for 72 hr., then poured into ice-water (20 ml.) and the resulting solutions were brought to pH 4-5 by the addition of sodium hydrogen carbonate. Acetylated sugars were extracted with chloroform and deacetylated with 0.5 N-barium methoxide as described in previous graded acetolysis experiments. The resulting mixtures of sugars were absorbed on charcoal columns (10 g.) and elution with water yielded mixtures of monosaccharides (galactose, arabinose, xylose and fucose and rhamnose in trace amounts), and subsequent elution of the columns with water containing 35% ethanol gave mixtures of oligosaccharides which were examined chromatographically in solvent A.

The data obtained from the examination of the fractions from the diethylaminoethylcellulose column is given in Table 7 and includes the relative proportions of sugars given on partial depolymerisation of the polysaccharide fractions.

It was noted that the polysaccharide, fraction 3, did not yield galactobiose or galactotriose on partial hydrolysis with 0.5 N-sulphuric acid for 4 hr. at 100°. A further partial hydrolysis of this polysaccharide with the same strength of acid but for half the hydrolysis period again failed to furnish these oligosaccharides as hydrolysis products, but partial hydrolysis with 0.1 N-sulphuric acid for 6 hr. yielded them in small amounts.

TABLE 7.

Fractions		1	2 (1st. Major)	3 (2nd. Major)	4	5
Wt. (mg.)		11.6	642	111	55.7	22.3
$[\alpha]_D$		+20°	+38°	+44°	+21°	+19°
U.A. %		—	16	36	—	—
Partial Hydrolysis Neutral Fraction	Gal$_3$	+	+	—	+	+
	Gal$_2$	3+	3+	—	3+	3+
	Gal.	5+	5+	2+	4+	4+
	Glu.	tr.	tr.	tr.	tr.	tr.
	Man.	—	tr.	tr.	tr.	—
	Ara.	+	5+	2+	4+	4+
	Xyl.	+	3+	5+	3+	—
	Fuc.	—	tr.	+	—	—
	Rha.	tr.	tr.	tr.	—	—
	MeS.	—	tr.	tr.	—	—
Acidic Fraction	GalA.	+	4+	5+	2+	+
R_{GalA}	0.78	—	3+	2+	2+	—
"	0.58	—	2+	+	+	—
"	0.22	—	2+	3+	+	—
"	0.11	—	2+	2+	2+	—
Acetolysis $R_{glucose}$	1.10		2+	+		
"	0.94		2+	+		
"	0.57		4+	3+		
"	0.30		2+	+		
"	0.10		+	—		

$R_{glucose}$ and R_{GalA} values are quoted for solvent A ; U.A. = Uronic Anhydride by decarboxylation.

MeS. = methylated sugar; tr. = trace.

Gal$_2$ and Gal$_3$ represents the oligosaccharides containing (1 → 4)-linked β-D-galactopyranose residues.

Large-scale Partial Hydrolysis of Soybean Meal Polysaccharide Extract ME2 with 0.5 N-Sulphuric Acid.

The polysaccharide (20 g.) was heated in 0.5 N-sulphuric acid (1 l.) on a boiling water bath for 4 hr. A small quantity (130 mg.) of degraded polysaccharide was separated at the centrifuge and gave on hydrolysis galacturonic acid (+++), glucose (++), galactose (trace), mannose (trace), xylose (+++), rhamnose (+), and methylated sugars (+). The hydrolysate was poured into acetone (1 vol.) and degraded polysaccharide (1.72 g.) which separated, gave on hydrolysis galacturonic acid (+++), galactose (trace), xylose (+++), rhamnose (+) and methylated sugars (+). The supernatant liquid was concentrated to remove acetone and sulphuric acid was neutralised by shaking with methyldi-n-octylamine (5% v/v in chloroform), and the aqueous solution was passed through Amberlite IR-120(H) resin and concentrated to a syrup (12.2 g.). The syrup was absorbed on charcoal (500 g.) and the squat column eluted with water (10 l.) to yield a mixture of monosaccharides (5.91 g.) chromatographically identified as rhamnose, xylose, arabinose, galactose, mannose and galacturonic acid.

Elution of the column with water containing 35% of ethanol furnished a syrup (3.42 g.) which contained a mixture of neutral and acidic oligosaccharides together with small amounts of two methylated sugars and traces of arabinose and galactose. The syrup was then absorbed on a column of diethylaminoethyl-Sephadex (formate form) (40 g.) and elution with water (3 l.) gave a mixture of sugars (1.65 g.) containing two methylated sugars, galactose, arabinose and a number of neutral oligosaccharides. Hydrolysis of a sample of this mixture gave the two methylated sugars, arabinose and a very much enlarged galactose content indicating that the neutral oligosaccharides were essentially composed of galactose moieties. Subsequent elution of the Sephadex column with 0.5 N-formic acid (4 l.) gave a mixture (0.91 g.) of acidic oligosaccharides R_{GalA} 0.75, 0.55, 0.40, 0.34, 0.28, 0.22 and 0.11, similar to those isolated in a later experiment.

The mixture (1.5 g.) of neutral sugars was added to a column of charcoal-Celite (1:1, 400 g.) and the column was eluted with water containing increasing proportions of ethanol. Fractions (25 ml.) were taken and a sample of every tenth tube was examined by chromatography in solvent B and the following eleven bulked fractions were obtained.

EXAMINATION OF NEUTRAL OLIGOSACCHARIDES

Fraction 1

The syrup (10 mg. ; eluted with water containing 1.0 - 5.0% of ethanol) contained traces of oligosaccharides and a sugar, $R_{glucose}$ 2.2 in solvent D, which was chromatographically identical to 2-_O_-methylxylose and gave xylose on demethylation with boron trichloride.

Fraction 2

The syrup (27 mg. ; eluted with water containing 1.0 - 5.0% of ethanol) contained traces of arabinose and galactose together with a sugar, $R_{glucose}$ 2.34 in solvent D, which was chromatographically identical to 2-_O_-methylfucose and gave only fucose on demethylation.

Fraction 3

The syrup (32 mg. ; eluted with water containing 1.0 - 5.0% of ethanol) contained traces of arabinose and galactose and a mixture of oligosaccharides of similar chromatographic mobility $R_{glucose}$ 0.43 and 0.40 in solvents B and D which stained yellow and red with _p_-anisidine hydrochloride. The mixture of oligosaccharides was separated from the monosaccharides by chromatography on filter sheets in solvent B, and on hydrolysis gave galactose and traces of arabinose and glucose.

Fraction 4

The chromatographically pure sugar (410 mg. ; eluted with water containing 5% of ethanol), M_G 0.48, $R_{glucose}$ 0.43 and $R_{galactose}$ 0.55 in solvent B, stained yellow with p-anisidine hydrochloride and gave galactose on hydrolysis. The sugar was crystallised from ethanol-water and after recrystallisation the crystals had m.p. and mixed m.p. 204°, $[\alpha]_D$ +90.6° (10 min.) $\longrightarrow$ +67.7° (equil.) (c 2.5 in water) and gave an X-ray powder photograph identical to an authentic sample of 4-O-β-D-galactopyranosyl-D-galactose.

The sugar (90 mg.) was methylated by shaking with methyl iodide (20 ml.), dimethylformamide (10 ml.) and silver oxide (5 g.) at room temperature in the dark for 24 hr. The mixture was filtered and the residue washed with chloroform and the filtrate and washings concentrated to yield partially methylated disaccharide. This procedure was repeated twice to give a fully methylated product (60 mg.) which was methanolysed with 3% methanolic hydrogen chloride (25 ml.) for 6 hr. at 100° and the resulting methyl glycosides were hydrolysed with 4% hydrochloric acid (20 ml.) for 3 hr. at 100°. The solution was neutralised by passage through a column of Amberlite IR-45(OH) resin and the eluate concentrated to a mixture of sugars which was chromatographed on filter sheets in solvent J to yield two sugar fractions.

The sugar (20 mg.) R_G 0.80 in solvent J, $[\alpha]_D$ +101° (c 1.0 in water) was chromatographically identical to 2,3,4,6-tetra-O-methyl-D-galactose and gave a characteristic aniline derivative m.p. and mixed m.p. 197-198°.

The sugar (21 mg.) R_G 0.53 in solvent J, $[\alpha]_D$ +82° (c 0.97 in water) was chromatographically identical to 2,3,6-tri-O-methyl-D-galactose and on oxidation with bromine-water gave a lactone m.p. 98° undepressed on admixture with an authentic sample of 2,3,6-tri-O-methyl-D-galactonolactone.

The disaccharide (50 mg.) in water (5 ml.) was oxidised with bromine (0.5 ml.) in the presence of barium carbonate (50 mg.). The mixture was shaken vigorously for 15 min. and stored for 72 hr. in the dark at room temperature with occasional shaking. The mixture was then aerated until colourless, filtered, and the barium ions removed from the resulting solution with Amberlite IR-120(H) resin. The solution was neutralised by shaking with Amberlite LA-2 resin (5% v/v in light petroleum) and concentrated to a syrup which was washed with chloroform to remove traces of liquid resin. The oxidised sugar was methylated by shaking with methyl iodide (10 ml.), dimethylformamide (10 ml.) and silver oxide (2.5 g.) at room temperature in the dark for 24 hr. The mixture was filtered and the residue washed with chloroform and the filtrate and washings concentrated to a syrup which was further methylated thrice to yield a fully methylated product (26 mg.), a sample of which was methanolysed and the products examined by gas chromatography and the results are shown in Table 8.

The fully-methylated oxidised product was methanolysed with 3% methanolic hydrogen chloride (10 ml.) for 6 hr. at 100° and the resulting methyl glycosides were hydrolysed with 4% hydrochloric acid (10 ml.) for 3 hr. at 100°. The resulting solution was neutralised with barium carbonate, filtered, concentrated to dryness and extracted with chloroform. The residue was dissolved in water and acidified with N-hydrochloric acid, evaporated to dryness and extracted with chloroform-ether mixtures. The chloroform extract was concentrated to a syrup which was paper chromatographically identified as 2,3,4,6-tetra-O-methyl-D-galactose and the chloroform-ether extract was found to contain a mixture of the tetramethylgalactose and 2,3,5,6-tetra-O-methyl-D-galactonolactone which was separated on a filter sheet in solvent H.

The 2,3,4,6-tetra-O-methyl-D-galactose, (8 mg.), R_G 0.97 in solvent H, gave a characteristic aniline derivative m.p. and mixed m.p. 197°.

The 2,3,5,6-tetra-O-methyl-D-galactonolactone, (6 mg.), R_G 1.12 in solvent H, was treated with methanolic ammonia (10 ml.) at -5° for 24 hr. The excess reagent was removed by evaporation to yield the amide which was recrystallised from alcohol and gave a m.p. and mixed m.p. 162°.

TABLE 8

Methyl glycosides	Relative Retention Time *T*	
	Column *b*	* Column *c*
2,3,4,6-tetra-*O*-methyl -*D*-galactose	1.56	1.80
2,3,5,6-tetra-*O*-methyl -*D*-galactonolactone	2.28	4.45

* Column *c* was 3% neopentylglycol adipate on Celite.

Fraction 5

The syrup (27 mg. ; eluted with water containing 10 - 12% of ethanol) contained the oligosaccharide in fraction 4 and a mixture of oligosaccharides of similar chromatographic mobility, $R_{glucose}$ 0.21, $R_{galactose}$ 0.27 in solvent B and $R_{glucose}$ 0.19 in solvent D, which stained yellow and red with *p*-anisidine hydrochloride, and gave on hydrolysis galactose and traces of arabinose and glucose.

Fraction 6

The chromatographically pure sugar (229 mg. ; eluted with water containing 12% of ethanol), $R_{glucose}$ 0.21 and $R_{galactose}$ 0.27 in solvent B, M_G 0.40, $[\alpha]_D$ +57.5° (*c* 1.1 in water), stained yellow with *p*-anisidine hydrochloride and gave only galactose on hydrolysis. Partial acid hydrolysis of the trisaccharide gave galactose and 4-*O*-β-D-galactopyranosyl-D-galactose.

The methanolysis products from the methylated trisaccharide were examined by gas chromatography and the following results shown in Table 9 were obtained.

TABLE 9.

Methyl glycosides	Relative Retention Time *T*	
	Column *a*	Column *b*
2,3,4,6-tetra-*O*-methyl-D-galactose	1.81	1.63
2,3,6-tri-*O*-methyl-D-galactose	3.17s 3.89vw 4.25w 4.6m	2.25w 2.55m 1.61s

A sample (10 mg.) of the trisaccharide in water (5 ml.) was oxidised with bromine (0.2 ml.) in the presence of barium carbonate (30 mg.). The mixture was stored for 72 hr. in the dark at room temperature, then aerated until colourless, filtered, deionised with Amberlite IR-120(H) resin and Amberlite LA-2 resin (5% v/v in light petroleum) and concentrated to a syrup which was methylated by three successive treatments with Kuhn reagents [methyl iodide (5 ml.), dimethylformamide (5 ml.) and silver oxide (1.5 g.)]. The methylated oxidised trisaccharide was methanolysed with 3% methanolic hydrogen chloride for 6 hr. at 100° and a portion of the methyl glycoside mixture was examined by gas chromatography which furnished the results shown in Table 10. The remaining mixture of methyl glycosides was hydrolysed with 4% hydrochloric acid (10 ml.) for 3 hr. at 100° and the resulting solution neutralised with barium carbonate, filtered, concentrated to dryness and extracted with boiling chloroform. The residue was dissolved in water, made acid with N-hydrochloric acid, evaporated to dryness and extracted with chloroform-ether mixtures. The chloroform extract was concentrated to a mixture of sugars, shown by chromatography in solvent H to contain 2,3,4,6-tetra- and 2,3,6-tri-O-methyl-D-galactose R_G 0.96 and 0.82 respectively. The chloroform-ether fraction was found to contain a trace of tetramethylgalactose and 2,3,5,6-tetra-O-methyl-D-galactonolactone R_G 1.12 which was detected with the hydroxylamine-ferric chloride spray reagent (23).

TABLE 10

Methyl glycosides	Relative Retention Time T	
	Column b	Column c
2,3,4,6-tetra-O-methyl -D-galactose	1.56	1.80
2,3,6-tri-O-methyl -D-galactose	1.56	3.00 4.24
2,3,5,6-tetra-O-methyl -D-galactonolactone	2.20	4.39

Column c was 3% neopentylglycol adipate on Celite.

Fraction 7

The syrup (81 mg. ; eluted with water containing 17.5 - 20% of ethanol) contained a main component $R_{glucose}$ 0.11, $R_{galactose}$ 0.14 in solvent B and $R_{galactose}$ 0.6 in solvent G, which stained yellow with p-anisidine hydrochloride, together with a trace of a second component of similar chromatographic mobility which stained red. Hydrolysis of the oligosaccharide mixture gave galactose and traces of arabinose and glucose.

Fraction 8

The chromatographically pure sugar (111 mg. ; eluted with water containing 20% of ethanol), $R_{glucose}$ 0.11, $R_{galactose}$ 0.14 in solvent B and $R_{galactose}$ 0.6 in solvent G, M_G 0.35, $[\alpha]_D$ +52° (c 1.0 in water), stained yellow with p-anisidine hydrochloride and on partial acid hydrolysis gave galactose, galactobiose and galactotriose. The tetrasaccharide was methylated and methanolysed and the resulting methyl glycosides were examined by gas chromatography and shown to have the retention times of methyl glycosides of 2,3,4,6-tetra- and 2,3,6-tri-O-methyl-D-galactose.

Fraction 9

The syrup (58 mg. ; eluted with water containing 30% ethanol) contained a mixture of sugars which was chromatographed on filter sheets in solvent G to give two fractions.

a) The minor fraction (10 mg.) was chromatographically and electrophoretically indistinguishable from galactotetraose found in fraction 8.

b) The major fraction (28 mg.), $R_{galactose}$ 0.07 and 0.45 in solvents B and G respectively, M_G 0.33, on partial acid hydrolysis gave galactose, galactobiose, galactotriose and galactotetraose. The methanolysis products of the methylated pentasaccharide were examined by gas chromatography and contained the methyl glycosides of 2,3,4,6-tetra- and 2,3,6-tri-O-methyl -D-galactose.

Fraction 10

The syrup (19 mg. ; eluted with water containing 30% ethanol) contained two oligosaccharides which were separated on filter sheets in solvent G.

a) The chromatographically pure sugar (4 mg.) $R_{galactose}$ 0.07 and 0.45 in solvents B and G was chromatographically and electrophoretically indistinguishable from galactopentaose (fraction 9b).

b) The chromatographically pure sugar (7 mg.) $R_{galactose}$ 0.04 and 0.34 in solvents B and G, M_G 0.31, on partial acid hydrolysis gave galactose, galactobiose, galactotriose, galactotetraose and galactopentaose. The methyl glycosides obtained from methanolysis of the methylated hexasaccharide were examined by gas chromatography and found to have the retention times of the methyl glycosides of

2,3,4,6-tetra- and 2,3,6-tri-O-methyl-D-galactose.

Fraction 11

The syrup (10 mg. ; eluted with water containing 30% ethanol) contained three oligosaccharides $R_{galactose}$ 0.34 (chromatographically similar to galactohexaose), $R_{galactose}$ 0.25 and $R_{galactose}$ 0.18 in solvent G. The syrup on partial acid hydrolysis gave galactose and the oligosaccharides mentioned in previous fractions. Gas chromatographic examination of the methyl glycosides from methanolysis of the methylated mixture of hexa-, hepta-, and octasaccharides indicated the presence of the methyl glycosides of 2,3,4,6-tetra- and 2,3,6-tri-O-methyl-D-galactose.

Large-scale Partial Hydrolysis of Soybean Meal Polysaccharide Extract ME2 with N-Sulphuric Acid.

Soybean meal polysaccharide (20 g.) was heated in N - sulphuric acid (1 l.) on the boiling-water bath for 7 hr. A small quantity (99 mg.) of degraded polysaccharide was removed at the centrifuge and on total hydrolysis yielded galacturonic acid (+++), glucose (+++), xylose (++) and rhamnose (+). The hydrolysate was poured into acetone (1 vol.) and the degraded polysaccharide (1.04 g.), which separated, gave on hydrolysis galacturonic acid with traces of xylose and rhamnose. The supernatant liquid was concentrated to remove acetone and the sulphuric acid was partially neutralised with saturated barium hydroxide solution. The precipitated barium sulphate was removed at the centrifuge and the supernatant completely neutralised by shaking with Amberlite LA-2 resin (5% v/v in light petroleum). The solution was then passed through a column of Amberlite IR-120(H) resin and finally concentrated to a syrup. The mixture of sugars was added to a charcoal column (500 g.) and elution with water gave a monosaccharide fraction (14 g.) containing galacturonic acid, galactose, xylose, arabinose, rhamnose and fucose. Elution of the column with water containing 35% of ethanol gave a syrup (3.65 g.) which was absorbed on a column of diethylaminoethyl-Sephadex A-25 (50 g. ; formate form).

Elution of the column with water gave a mixture (705 mg.) of neutral sugars including galactose and arabinose, two methylated sugars, and galactose-containing oligosaccharides identical to those mentioned in the previous experiment. The column was then eluted with formic acid solutions of increasing strength to give the fractions shown in Table 11, and further fractionation was carried out by chromatography on Whatman filter sheets using solvents A and C.

TABLE 11.

Fract -ion	Wt. (mg.)	Formic Acid in Eluant	Component Sugars	Chromatography (R_{GalA})		Relative Amounts
				Solvent A	Solvent C	
1	43	0.05 N	GalA.	1.00	1.00	++
			O_1	0.78	1.00	++++
2	131	0.05 N	O_1	0.78	1.00	
			Sugar	0.46	0.79	tr.
3	177	0.05 N	O_1	0.78	1.00	+++++
			Sugar	0.46	0.79	+
4	61	0.05 N	GalA.	1.00	1.00	++
			O_1	0.78	1.00	+++
			Sugar	0.46	0.79	++
			O_2	0.20	0.55	++
			Sugar	0.05	0.31	++
5	63	0.05 N	GalA.	1.00	1.00	+
			O_1	0.78	1.00	+
			O_3	0.58	0.93	++
			O_2	0.20	0.55	++++
6	78	0.05 N	GalA.	1.00	1.00	tr.
			O_1	0.78	1.00	tr.
			O_3	0.58	0.93	+++
			O_4	0.29	0.69	+
			O_2	0.20	0.55	+

TABLE 11 (contd.)

Fraction	Wt. (mg.)	Formic Acid in Eluant	Component Sugars	Chromatography (R_{GalA})		Relative Amounts
				Solvent A	Solvent C	
7	18	0.05-0.4 N	GalA.	1.00	1.00	+++
			O_1	0.78	1.00	++
8	196	0.4 N 2%	O_5	0.11	0.60	
			Sugar	—	0.13	tr.
9	18	0.4 N	O_6	0.12	0.48	
			Sugar	—	0.13	tr.
10	54	0.4-0.5 N	O_7	0.23	0.67	+++
			O_6	0.12	0.48	+
			Sugar	—	0.13	+
11	39	0.4-0.5 N	O_7	0.23	0.67	
			Sugar	—	0.13	tr.
12	81	0.4-0.5 N	O_7	0.23	0.67	tr.
			O_8	0.20	0.51	+++
			O_9	0.05	0.22	tr.
13	40	0.5 N	O_8	0.20	0.51	+++
			O_9	0.05	0.22	++
14	182	0.5 N	O_9	0.05	0.22	+++
			+ Higher Oligo's.		0.20 → Streak	++++

EXAMINATION OF ACIDIC OLIGOSACCHARIDES

Oligosaccharide 1 (O_1)

280 mg.

R_{GalA} = 0.78 in solvent A

M_G = 0.59

The sugar, $[\alpha]_D$ +86° (c 0.9 in water), which stained orange-red with p-anisidine hydrochloride, gave galacturonic acid and rhamnose on hydrolysis and was chromatographically and electrophoretically indistinguishable from 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose. A sample (50 mg.) of the sugar was methylated by six additions of methyl sulphate (2 ml. each) and 30% aqueous sodium hydroxide (6 ml. each). The resulting solution was heated to 80° for 30 min., allowed to cool and made acid to pH 3.0 by the addition of dilute sulphuric acid. The acidified solution was extracted with chloroform (3 x 20 ml.) and the combined chloroform solutions concentrated to give methylated disaccharide which was crystallised and recrystallised from a chloroform-petroleum (b.p. 100-120°) mixture containing a trace of water. The crystals, $[\alpha]_D$ +93° (c 1.1 in chloroform) had a m.p. and mixed m.p. 70° (Kofler hot-stage microscope), m.p. and mixed m.p. 120° (capillary tube) and gave an X-ray powder photograph identical to that of methyl 2-O-(2,3,4-tri-O-methyl-D-galactopyranosyluronic acid)-3,4-di-O-methyl-L-rhamnose dihydrate (99).

Oligosaccharide 2 (O_2)

8 mg.
R_{GalA} = 0.20 in solvent A
M_G = 1.0

The sugar, $[\alpha]_D$ +4° (*c* 0.6 in water), which stained yellow with *p*-anisidine hydrochloride, gave galactose, glucuronic acid and glucuronolactone on hydrolysis and was chromatographically and electrophoretically indistinguishable from 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose.

The disaccharide was methylated by the small-scale Kuhn method (general methods) and the products of methanolysis examined by gas chromatography. The results obtained are shown in Table 12.

TABLE 12.

Methyl glycosides	Relative Retention Time *T*	
	Column *a*	Column *b*
2,3,4-tri-*O*-methyl-D-glucuronic acid*	2.49 3.20	1.77 2.20
2,3,4-tri-*O*-methyl-D-galactose	7.2	2.62 2.91
2,3,5-tri-*O*-methyl-D-galactose	4.43	1.98

* Present as the methyl ester.

Oligosaccharide 3 (O_3)

16 mg.
R_{GalA} = 0.58 in solvent A
M_G = 0.58

The sugar, $[\alpha]_D$ -68° (*c* 1.47 in water), stained yellow with *p*-anisidine hydrochloride and gave fucose, glucuronic acid and glucuronolactone on hydrolysis. The disaccharide was methylated and the products of methanolysis examined by gas chromatography. The following Table 13 indicates the retention times of the peaks, but the lack of authentic dimethyl fucose standards meant that the chromatograms could not be fully interpreted.

TABLE 13.

Methyl glycosides	Relative Retention Time *T*	
	Column *a*	Column *b*
2,3,4-tri-*O*-methyl -*D*-glucuronic acid*	2.51m 3.22s	1.77m 2.21s
? dimethyl fucose	1.10-1.11m 1.56s 2.04vw	0.60m 0.93s 1.29vw

* Methyl glycoside present as the methyl ester.

Authentic samples of sugars together with the disaccharide and the derived glycitol (borohydride reduction) were applied to Whatman No. 1 paper, sprayed with a 1% aqueous solution of sodium metaperiodate, left for 5 min., treated with sulphur dioxide and lightly sprayed with a Schiff's reagent prepared by dissolving pararosaniline hydrochloride (1 g.) in water (100 ml.) which had been saturated with sulphur dioxide. The colours developed by the action of the spray reagent (84) on the following sugars were:-

Sugar	Colour
2-*O*-methylfucose	Yellow
2-*O*-(α-*D*-galactopyranosyluronic acid)-*L*-rhamnose	Yellow
2-*O*-(α-*D*-galactopyranosyluronic acid)-*L*-rhamnitol	Blue
3-*O*-α-*D*-glucopyranosyl-*L*-arabinose	Yellow
3-*O*-β-*D*-glucopyranosyl-*D*-glucitol (Laminaribiitol)	Yellow
Glucuronosylfucose (O_3)	Blue
Glucuronosylfucitol (O_3 reduced)	Blue

A sample of the oligosaccharide was spotted on filter paper and sprayed with a 0.5% chloroform solution of 2,3,5-triphenyltetrazolium chloride then with methanolic sodium hydroxide; a bright red colour developed indicating the absence of a 2-*O*-substituted reducing sugar (21). The probable structure of the disaccharide based on the evidence given was 4-*O*-(β-*D*-glucopyranosyluronic acid)-*L*-fucose.

Oligosaccharide 4 (O_4)

4 mg.
R_{GalA} = 0.29 in solvent A
M_G = 0.67

The sugar, $[\alpha]_D$ -28° (c 0.18 in water) stained yellow with p-anisidine hydrochloride and gave galactose, glucuronic acid and glucuronolactone on hydrolysis. The methanolysis products of the methylated disaccharide were examined by gas chromatography and the results shown in Table 14 indicated that the aldobiouronic acid was 4-O-(β-D-glucopyranosyluronic acid)-D-galactose.

TABLE 14

Methyl glycosides	Relative Retention Time T	
	Column a	Column b
2,3,4-tri-O-methyl-D-glucuronic acid*	2.50 3.21	1.76 2.22
2,3,6-tri-O-methyl-D-galactose	4.25 3.21 4.60	1.64 2.55 2.23

* Present as the methyl ester.

Oligosaccharide 5 (O_5)

105 mg.
R_{GalA} = 0.11 in solvent A
M_G = 0.67

The sugar, $[\alpha]_D$ +104° (c 0.93 in water), which stained orange-red with p-anisidine hydrochloride gave on hydrolysis galacturonic acid and rhamnose in the molar proportion of 1:1, and after reduction with potassium borohydride the ratio was 1.7:1 ; the estimations were performed by the carbazole (37) and L-cysteine hydrochloride (39) methods.

The tetrasaccharide on partial acid hydrolysis gave galacturonic acid, rhamnose, and 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose and these sugars together with rhamnitol and 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnitol were detected in the partial acid hydrolysate of the derived glycitol (borohydride reduction). The 2-O-(α-D-galactopyranosyluronic acid) -L-rhamnose R_{GalA} 0.58 and the corresponding glycitol R_{GalA} 0.73 were effectively separated on Whatman No. 4 paper in solvent I and were detected with silver nitrate reagent after the chromatogram had been sprayed with methanol and heated in an oven to remove the boric acid.

A sample of the tetrasaccharide (60 mg.) was methylated by three treatments with Kuhn reagents [methyl iodide (25 ml.) dimethylformamide (20 ml.) and silver oxide (3 g.)] and a sample of the methylated sugar was methanolysed and the resulting mixture of methyl glycosides was examined by gas chromatography and gave the results shown in Table 15.

The remainder of the methylated tetrasaccharide (39 mg.) was dissolved in tetrahydrofuran (25 ml.) and at intervals aluminium lithium hydride (50 mg.) was added to the refluxing solution over a period of 2 hr. The excess hydride was destroyed by adding ethyl acetate followed by water and the resulting mixture was extracted with chloroform several times and the extracts were combined, dried and concentrated to yield a reduced methylated product (30 mg.)

A sample of the reduced methylated product (25 mg.) was methanolysed with 3% methanolic hydrogen chloride (10 ml.) for 6 hr. at 100° and a small portion of the resulting methyl glycoside mixture was examined by gas chromatography and furnished the results shown in Table 15. The remainder of the methyl glycoside mixture was hydrolysed with 4% hydrochloric acid (10 ml.) for 3 hr. at 100°, the resulting solution was neutralised by passage through a column of Amberlite IR-45(OH) resin and the eluate concentrated to give a mixture of sugars shown by paper chromatography in solvent H to contain 3,4-di-*O*-methyl-*L*-rhamnose R_G 0.87, 2,3,4-tri- and 2,3-di-*O*-methyl-*D*-galactose R_G 0.70 and 0.52 respectively. The 2,3-di-*O*-methyl-*D*-galactose, $R_{rhamnose}$ 1.91, was distinguishable from 2,4-di- and 2,6-di-*O*-methyl-*D*-galactose, $R_{rhamnose}$ 1.47 and 2.12 respectively, in solvent J the chromatogram having been given three 9 hr. irrigations.

The remaining reduced methylated tetrasaccharide (5 mg.) was remethylated by treatment with Kuhn reagents, the remethylated product methanolysed with 3% methanolic hydrogen chloride and the resulting mixture of methyl glycosides examined by gas chromatography to give the results shown in Table 15. The presence, as indicated by gas chromatography, of the methyl glycoside of 2,3,4-tri-*O*-methyl-*D*-galactose in the methanolysis products indicated that probably incomplete remethylation had occurred.

TABLE 15

Methyl glycosides	Relative Retention Time *T*	
	Column *b*	Column *c*
(METHYLATED)		
3,4-di-*O*-methyl-*L*-rhamnose	0.61	0.88
2,3,4-tri-*O*-methyl-*D*-galacturonic acid*	4.17 3.81	6.62 6.22
2,3-di-*O*-methyl-*D*-galacturonic acid*	2.17	4.95
(REDUCED METHYLATED)		
3,4-di-*O*-methyl-*L*-rhamnose	0.62	0.89
2,3,4-tri-*O*-methyl-*D*-galactose	2.80 2.54	6.94
2,3-di-*O*-methyl-*D*-galactose	—	—
(REMETHYLATED REDUCED METHYLATED)		
3,4-di-*O*-methyl-*L*-rhamnose	0.61	0.87
2,3,4,6-tetra-*O*-methyl-*D*-galactose	1.58	1.80
2,3,6-tri-*O*-methyl-*D*-galactose	1.58	2.99
2,3,4-tri-*O*-methyl-*D*-galactose	2.80 2.53	6.93

* Present as methyl esters.

Column *c* was 3% neopentylglycol adipate, on Celite.

This evidence was consistent with the structure O-α-D-galactopyranosyluronic acid-(1⟶2)-O-β-L-rhamnopyranosyl-(1⟶4)-O-α-D-galactopyranosyluronic acid-(1⟶2)-L-rhamnopyranose.

Oligosaccharide 6 (O_6)

10 mg.

$R_{GalA} = 0.48$ in solvent C

$M_G = 0.81$

The syrup, $[\alpha]_D$ +70° (c 0.88 in water), which stained orange-red with p-anisidine hydrochloride, gave galacturonic acid and rhamnose on hydrolysis in the molar proportion of 1.8:1 and after reduction with potassium borohydride the ratio was 1.2:1. On partial acid hydrolysis the fraction yielded galacturonic acid, rhamnose and 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose and these sugars together with a small amount of rhamnitol on hydrolysis of the potassium borohydride reduced oligosaccharide fraction. The fraction was methylated with Kuhn reagents in the manner described for oligosaccharide 5 and the products of methanolysis were shown by gas chromatography to be essentially the methyl esters methyl glycosides of 2,3-di- and 2,3,4-tri-O-methyl-D-galacturonic acid together with a trace of the methyl glycoside of 3,4-di-O-methyl-L-rhamnose ; the chromatogram also contained substantial amounts of other peaks which could not be identified. Towards the end of the investigation on this fraction it was discovered that although the fraction appeared chromatographically homogeneous on Whatman No. 1 paper in solvents A and C, a sample run on Whatman No. 4 paper in solvent A for two days indicated the presence of two oligosaccharides which were incompletely separated, but the more mobile component was chromatographically identical to oligosaccharide 5.

Oligosaccharide 7 (O_7)

12 mg.

R_{GalA} = 0.67 in solvent C

M_G = 0.60

The sugar, $[\alpha]_D$ +87° (c 1.12 in water), which stained crimson with p-anisidine hydrochloride, gave galacturonic acid and rhamnose on hydrolysis in the molar proportion of 1.2:1 and after reduction with potassium borohydride the ratio was 2.3:1. On partial acid hydrolysis the sugar yielded galacturonic acid, rhamnose and 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose and these sugars together with rhamnitol on hydrolysis of the derived glycitol (borohydride reduction). The sugar was methylated with Kuhn reagents in the manner described for oligosaccharide 5 and the products of methanolysis were shown by gas chromatography to be principally the methyl ester methyl glycoside of 2,3,4-tri-O-methyl-D-galacturonic acid and the methyl glycoside of 3,4-di-O-methyl-L-rhamnose together with a number of other compounds giving peaks which could not be identified. A very complex gas chromatogram, which could not be interpreted, was obtained from the methanolysis products of the remethylated reduced methylated oligosaccharide.

Oligosaccharide 8 (O_8)

	45 mg.
R_{GalA}	0.51 in solvent C
M_G	0.92

The sugar, which stained crimson with p-anisidine hydrochloride, gave on hydrolysis galacturonic acid and was chromatographically and electrophoretically indistinguishable from 4-O-α-D-galactopyranosyluronic acid-D-galactopyranosyluronic acid. The acid (15 mg.) in water (5 ml.) was neutralised with calcium carbonate, the excess solid was removed by filtration, and by the addition of acetone (2 vol.) to the filtrate the calcium salt precipitated, was washed with acetone and ether and dried to give a white solid $[\alpha]_D$ +113° (c 0.98 in 0.5N-hydrochloric acid)(99).

Oligosaccharide 9 (O_9)

	80 mg.
R_{GalA}	0.22 in solvent C
M_G	0.90

The sugar, which stained crimson with p-anisidine hydrochloride, gave on hydrolysis galacturonic acid and was chromatographically and electrophoretically indistinguishable from O-α-D-galactopyranosyluronic acid-(1→4)-O-α-D-galactopyranosyluronic acid-(1→4)-D-galactopyranosyluronic acid. The acid (30 mg.) in water (10 ml.) was neutralised with calcium carbonate, the excess solid removed by filtration, and by the addition of acetone the calcium trigalacturonate was precipitated, separated, washed with acetone and ether and dried. The salt was reprecipitated twice to give a white solid, $[\alpha]_D$ +140° (c 1.0 in 0.5 N-hydrochloric acid) (99).

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