PART - III

STUDIES ON A NEUTRAL POLYSACCHARIDE ISOLATED FROM BAEL FRUIT PULP

RESULTS AND DISCUSSION

Extraction of crude polysaccharide from pulp of bael (*Aegle marmelos*) fruit and characterization of constituent sugars

The bael fruit pulp was collected from well-developed, but unripe bael fruit by removing the rinds and seeds, along with the gummy envelope, and then macerated under ethanol, squeezing out the solvent and air-drying the material. Polysaccharide A was isolated from a hot ammonium oxalate extract of the powdered pulp by precipitation with ethanol, it had $[\alpha]_D^{23}$ + 116°. Under the same conditions, the hot aqueous extract of the pulp yielded polysaccharide B, $[\alpha]_D^{23}$ + 101°.

Polysaccharide A was hydrolyzed with 1M sulfuric acid. The hydrolyzate, on paper chromatographic examination showed spots for galactose, glucose, arabinose, rhamnose, galacturonic acid and a slower moving spot (faint). The presence of galacturonic acid was also confirmed by preparing carboxyl-reduced polysaccharide, hydrolyzing the product, and then analyzing the contents of the hydrolyzate by g.l.c.

Polysaccharide A was mixed with *myo*-inositol (used as the internal standard) and after hydrolysis, the component sugars were estimated, as alditol acetates, by g.l.c. The

mixture contained galactose (12.70%), glucose (19.37%), arabinose (10.75%), rhamnose (2.15%). The galacturonic acid was estimated by carbazole-sulfuric acid method[43] and was found to be 53.50%.

Polysaccharide B was found to contain the same monosaccharide residues present in polysaccharide A.

High-voltage electrophoreses of each of these polysaccharides showed heterogeneity giving two spots with some tailing in each case. Polysaccharide A was used for fraction and other investigations.

Fractionation of polysaccharide A with calcium chloride

In order to obtain a homogeneous polysaccharide, polysaccharide A was dispersed in water. The suspension was clear when made ammoniacal (pH 8.5) and then treated with aqueous calcium chloride (5%). The calcium pectate precipitated, was removed off, and the supernatant liquor was treated with cold ethanol, to yield a precipitate of polysaccharide C, which showed $[\alpha]_D^{23}$ + 53.5° and contained all of the monosaccharide residues present in polysaccharide A. Polysaccharide C was also heterogeneous according to high-voltage electrophoresis and contained 47.10% of galacturonic acid (by carbazole-sulfuric acid method[43]).

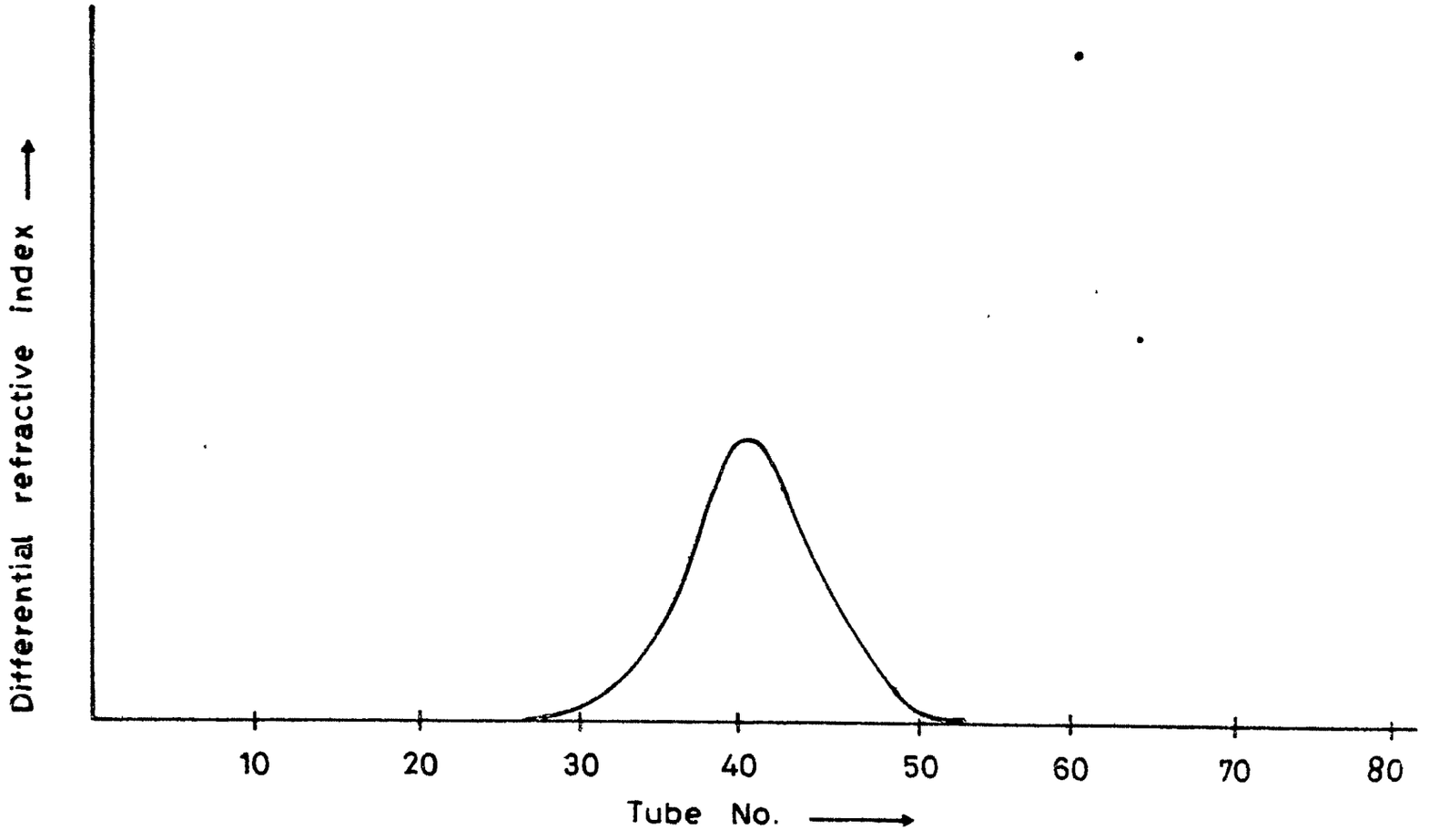
Resolution of polysaccharide C on a DEAE-cellulose column

To achieve further fractionation, polysaccharide C was resolved on a column of DEAE-cellulose which was eluted successively with (i) water, and (ii) 1.0M sodium chloride solution, the elution being monitored by means of 1-naphthol, to yield two polysaccharide fractions, polysaccharide D and polysaccharide E (see experimental).

Polysaccharide E was still heterogeneous (according to high-voltage, electrophoresis), and contained all of the monosaccharide residues present in polysaccharide A or C. Polysaccharide D was found to be homogeneous by high-voltage electrophoresis using borate buffer (0.02M, pH 9.28) and phosphate buffer (0.05M, pH 7.8).

Attempted fractionation of polysaccharide D, on column of Sephadex G-100

A solution of polysaccharide D in ammonium hydrogencarbonate buffer (pH 8.17) was passed through a column (95 x 1.1 cm) of Sephadex G-100 and eluted with the same buffer. The polysaccharide was recovered as a single peak (Fig. 2) in 95.3% yield. This also proved further that the polysaccharide D was homogeneous.



Column chromatography of Polysaccharide D using Sephadex G-100.

Figure 2.

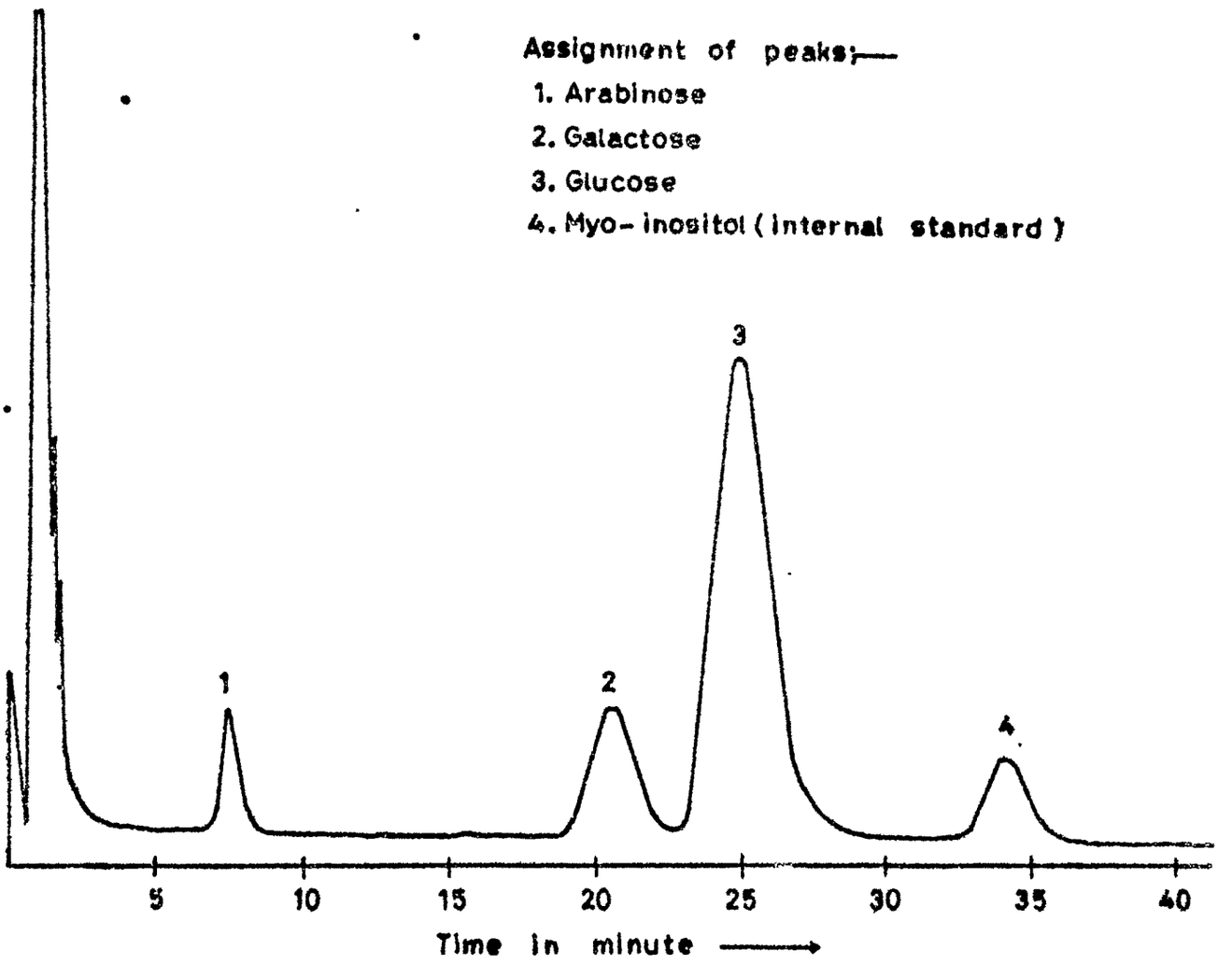
Hydrolysis of polysaccharide D and estimation of sugar components

Polysaccharide D was hydrolyzed with 1M sulfuric acid using myo-inositol as the internal standard and analyzed as alditol acetates, by g.l.c. The hydrolyzate contained arabinose (10.8%), galactose (16.4%), and glucose (71.4%), (Figure 3); rhamnose and galacturonic acid were absent, as shown by paper-chromatography, g.l.c. and use of the carbazole-sulfuric acid reagent.

Methylation analysis of polysaccharide D

Polysaccharide D was methylated first by Hakomori method[45] and then by Purdie method[47]. The methylated product had $[\alpha]_D^{23}$ + 56° and showed no O-H absorption band in its i.r. spectrum. It was hydrolyzed as stated in part II. The acid was neutralized with barium carbonate, and the partially methylated sugars were converted into their alditol acetates, and these analyzed by g.l.c. The results are shown in Table 5.

These results gave an indication as to the linkages between the different monosaccharide residues in polysaccharide D. Identification of 2,3,5-tri-O-methylarabinose and of 2,3,4,6-tetra-O-methyl-glucose and -galactose indicated their presence as the nonreducing end-units in the methylated polysaccharide;



Gas liquid chromatogram of alditol acetates of sugars obtained from Polysaccharide D.

Figure 3.

Table - 5

Methyl ethers of sugars from the hydrolyzates of methylated, neutral polysaccharide.

Sugars*	T+	Approximate mole %	Mode of linkages
2,3,5-Ara	0.43	4.85	Araf-(1→
3,5-Ara	0.78	4.75	→2)-Araf-(1→
2,3,4,6-Glc	1.00	4.89	Glc p -(1→
2,3,6-Glc	2.30	54.89	→4)-Glc p -(1→
2,3-Glc	4.47	11.91	→4,6)-Glc p -(1→
2,3,4,6-Gal	1.17	6.07	Gal p -(1→
2,3,6-Gal	2.21	5.17	→4)-Gal p -(1→
2,6-Gal	3.17	5.03	→3,4)-Gal p -(1→

* 2,3,5-Ara = 2,3,5-tri-O-methylarabinose, etc. Traces of 2,5-Ara, 2,4,6-Gal, and 6-Glc were also detected.

+ Retention times of the corresponding alditol acetates, relative to that of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol, on a 1% OV-225 column at 173°.

arabinose residues have the furanosyl structure and the other two have pyranosyl structure. In the interior part of the polysaccharide, both glucose and galactose are present as (1→4)-linked residues; of them, the former are preponderant, as a larger amount of 2,3,6-tri-O-methylglucose than of 2,3,6-tri-O-methylgalactose was found. The presence of 3,5-di-O-methylarabinose in the hydrolyzate of the methylated polysaccharide showed that arabinosyl groups are joined through (1→2)-linkages. Branch points in the polysaccharide originate from both glucose and galactose residues. Identification of 2,3-di-O-methylglucose and 2,6-di-O-methylgalactose indicated that, at branch points, glucose residues are linked through O-1, O-4 and O-6, whereas galactose residues are linked through O-1, O-3, and O-4. Traces of 2,5-di-O-methylarabinose, 2,4,6-tri-O-methylgalactose, and 6-O-methylglucose, which were also detected in the hydrolyzate of methylated polysaccharide D, possibly play no significant role in the chemical architecture of methylated macromolecule.

Smith[79] degradation of polysaccharide D

Polysaccharide D was subjected to Smith[79] degradation. The degraded products were converted into alditol acetates whose analysis by g.l.c. using column (a) indicated the presence of alditol acetates of threose, erythrose, arabinose, and galactose in the molar ratios of 1 : 10.8 : 1 : 1, together with glycerol

(which could not be correctly estimated). The large amounts of erythritol originated from (1⟶4)-linked glucosyl residues, which were expected to yield ~13, rather than 10.8, molar proportions of erythritol; this discrepancy could not, however, be explained. The equimolar proportions of threitol, arabinose, and galactose originated from (1⟶4)-linked galactose, (1⟶2)-linked arabinofuranose, and the branched galactosyl residues, respectively. In addition, a slight trace of glucose was detected in the Smith-degradation product; which might have originated from the 6-O-methylglucose identified in the hydrolyzate of the methylated polysaccharide D. Hence the above results supported the data obtained from methylation analysis of the polysaccharide D.

Chromium trioxide oxidation[80,81] of polysaccharide D

In order to ascertain the anomeric configurations of different sugar residues, the acetylated polysaccharide D was subjected to oxidation with chromium (VI) trioxide[80,81] in acetic acid at 50°, for various intervals of time, using *myo*-inositol as the internal standard. The aliquots at 0 hour, 1 hour and 2 hour were withdrawn and treated as described earlier. The surviving sugars were estimated by g.l.c. (as their alditol acetates). The results are shown in Table 6. The rapid disappearance of galactose during the reaction indicated that the

Table - 6

Survival of sugars* in oxidation of acetylated, neutral polysaccharide with chromium trioxide

Time (h)	Myo-inositol	Glucose	Galactose	Arabinose
0	10	365	49	31
1	10	149	0	29
2	10	147	0	19

* The sugars were analyzed, and estimated by g.l.c. using column (a) at 190°.

galactosyl groups had the β-configuration. The rate at which glucose was oxidized revealed the presence of both ∝- and β-configurations in glucosyl residues. Because, in the first hour, there was rapid oxidation, but then the rate was very slow. As acetylated furonoses are nonspecifically oxidized[80] by chromium trioxide, the anomeric configuration of the arabinofuranosyl groups could not be determined by this experiment. In fact, the rate at which arabinose was hydrolyzed off with 5% formic acid during 2 hours at 100° indicated that it had the ∝-configuration.

Graded hydrolysis studies on polysaccharide D

Polysaccharide D was subjected to graded hydrolysis with 40% formic acid for 4 hours at 100°, the optimal conditions having been found out with the aid of pilot experiments. It showed spots for monosaccharides and a few spots in the region of oligosaccharides on the paper chromatogram. The oligosaccharides were resolved and isolated by preparative paper-chromatography. After repeated chromatography on papers only two oligosaccharides were obtained in pure state.

Oligosaccharide I, $[\alpha]_D^{23}$ + 32.2°, on hydrolysis and analysis of the alditol acetates of the hydrolyzate by g.l.c. showed peak for glucose only. Methylation, hydrolysis and analysis of the alditol acetates by g.l.c. showed peaks for

2,3,4,6-tetra-O-methylglucose and 2,3,6-tri-O-methylglucose in almost equimolar proportions (1.06 : 1). From the above data oligosaccharide I appears to have a (1⟶4)-linked glucobiose structure. The specific rotation value (+ 32.2°) further suggests that the disaccharide has cellobiose structure and not maltose ($[\alpha]_D$ + 128°). Polysaccharide D has glucosyl groups of both α- and β-configurations, but it appears that the more stable disaccharide cellobiose could be isolated under this hydrolytic condition. Hence the correct structure of the oligosaccharide I is found to be as shown in (12).

β - Glc - (1⟶4) - Glc

(12)

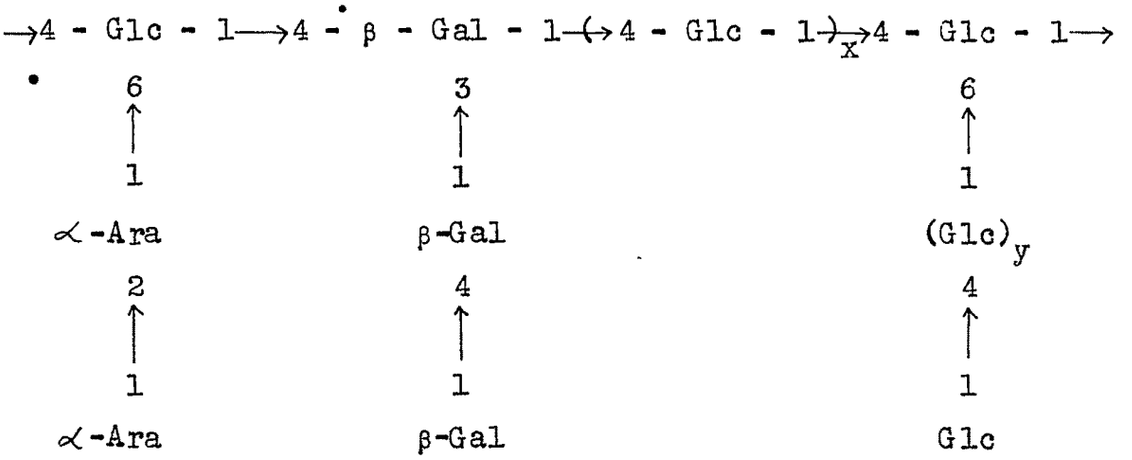
Oligosaccharide II, $[\alpha]_D^{23}$ + 60.7°, Lit[121] + 68°, showed a peak for galactose on g.l.c. analysis of the alditol acetates of its hydrolyzate. Methylated oligomer was hydrolyzed and after preparing the alditol acetates, was analyzed by g.l.c. It showed peaks for 2,3,4,6-tetra-O-methylgalactose and 2,3,6-tri-O-methylgalactose in almost equimolar proportions (1.03 : 1). From all these data structure (13) may be assigned to this oligomer.

β - Gal - (1⟶4) - Gal

(13)

CONCLUSIONS

Results of sugar analysis, methylation studies, Smith degradation and analysis of oligosaccharides are reported in this section. Consideration of all these experimental results the general structure (14) may be assigned to the average repeating unit of the polysaccharide D. This structure clearly indicates the presence of a basal chain of (1⟶4)-linked glucopyranose and galactopyranose residues from some of which branches originate. It also correctly reveals the type of linkages among the different sugar residues. However, this structure probably does not depict the correct sequence of branches. It is also not possible from the available data to assign the anomeric nature of the glucopyranosyl groups.



(14)

where x + y = 11, Glc = a glucopyranosyl, Gal = a galactopyranosyl, and Ara = an arabinofuranosyl group.

EXPERIMENTAL

Extraction of polysaccharide from fruit pulp

Twenty well-grown, but unripe, bael fruits (average weight, 500 gm each) were cut up. Pulp was isolated by removing the rinds and seeds, along with the gummy envelope. The pulp was then macerated under ethanol in a blender. The pulpy material was filtered with a piece of nylon cloth, and air dried. Yield 130 gm.

One part of the powdered pulp (60 gm) was suspended in 0.5% ammonium oxalate solution (1 litre) and heated for 4 hours at 85^{o} on a water bath with stirring. The extract was squeezed through a piece of nylon cloth and centrifuged at 6000 r.p.m. for 30 minutes for clarification. The process of extraction on the residue was repeated (in 0.5% ammonium oxalate solution, 500 ml). The cold, supernatant liquors were combined, acidified with acetic acid (pH 4.5) and cold ethanol (3 volumes) was added to this acidified solution. The light brown precipitate was collected by centrifugation (6000 r.p.m., 30 minutes), washed three times with ethanol, dissolved in water (600 ml), and reprecipitated with ethanol. The process of dissolution and precipitation was repeated once. The final precipitate

(Polysaccharide A) was washed with dry ethanol (3 times), and dried *in vacuo*; yield 7.74 gm, $[\alpha]_D^{23}$ + 116° (c 0.56, water). Another batch of this polysaccharide A was made with 30 gm of the pulp.

Another part of the powdered pulp (30 gm) was extracted with water (750 ml) for 4 hours at 85° by following the same procedure as just described. Polysaccharide B was precipitated with ethanol (3 volumes), washed and dried; yield 5.5 gm, $[\alpha]_D^{23}$ + 101° (c 0.6, water).

Hydrolysis of the polysaccharides A and B, and identification of the sugar components

Polysaccharides A and B (10 mg each) were hydrolyzed with 1M sulfuric acid for 20 hours on a boiling-water bath. The solutions were cooled, made neutral with $BaCO_3$, and centrifuged. Parts of the centrifugates were analyzed by paper chromatography using solvent systems (A), (B) and (C) and spray reagent (1), when spots corresponding to rhamnose, arabinose, glucose, galactose, galacturonic acid and a faint spot near the base line were detected. The other parts of the hydrolyzates were converted into their alditol acetates and examined by g.l.c. using column (a) at 190°. The chromatograms showed peaks for rhamnose, arabinose, glucose and galactose.

Estimation of sugar components

Polysaccharide A (5.3 mg) was mixed with myo-inositol (1.2 mg) and hydrolyzed with 1M sulfuric acid for 20 hours at 100°. The hydrolyzate after the usual treatment, was converted into alditol acetates and analyzed by g.l.c. using column (a) at 190°. Polysaccharide A contained galactose (12.70%), glucose (19.37%), arabinose (10.75%) and rhamnose (2.15%). The galacturonic acid was estimated by carbazole-sulfuric acid method[43] (using D-galacturonic acid as the standard) and was found to be 53.50%.

Fractionation of the polysaccharide A with calcium chloride

Polysaccharide A (5 gm) was dispersed in water (500 ml) by magnetic stirring for 1 hour. The suspension was made ammoniacal (pH 8.5) with dilute ammonia, and stirring was continued for 4 hours at room temperature, to afford a clear solution. The pectate was precipitated by dropwise addition of 5% calcium chloride (65 ml). After addition was complete, the suspension was stirred for another 3 hours at room temperature, when the turbidity gradually increased. The precipitate was allowed to settle overnight at 10°. The mixture was centrifuged (6000 r.p.m. for 30 minutes) and the supernatant liquor was collected. The precipitate was washed several times with

water and the washings were combined with the supernatant liquor. The combined supernatant was dialyzed for 2 days against distilled water, concentrated to ~ 300 ml, and then cooled to 5°. The contents were precipitated by slow addition of ethanol (4 volumes). The precipitate was collected by centrifugation (6000 r.p.m., 30 minutes), washed (twice) with absolute ethanol and the brownish material (polysaccharide C) air-dried; yield 1.156 gm, $[\alpha]_D^{23}$ + 53.5° (c 1.01, water).

Resolution of polysaccharide C on a DEAE-cellulose column

Polysaccharide C (500 mg) was dissolved in water (25 ml) and decationized by passing the solution through a column (12 x 1.5 cm) of Dowex-50W X-8(H^+) resin. The column was washed, and eluted, with water. The eluate and washings were combined, concentrated and lyophilized to yield ~ 360 mg of material. This decationized material was dissolved in water (25 ml) and was added to the top of a column (20 x 2.5 cm) of DEAE-cellulose. The column was successively eluted with water and 1.0M sodium chloride solution, elution being monitored with 1-naphthol in presence of sulfuric acid. The fractions containing carbohydrate materials were pooled. The fraction obtained by elution with water was concentrated, and lyophilized, to give polysaccharide D; yield 35.6 mg, $[\alpha]_D^{23}$ + 65.9° (c 0.5, water). The fraction obtained from sodium chloride eluate was dialyzed against

distilled water for 2 days to remove salt. The salt free solution was concentrated and then lyophilized to give polysaccharide E; yield 91.7 mg, $[\alpha]_D^{23}$ + 33° (c 0.6, water).

High-voltage electrophoresis of different polysaccharide fractions

High-voltage electrophoreses were performed at 70 V. cm^{-1} for 1 hour, using (i) borate buffer (0.02M, pH 9.28) and (ii) phosphate buffer (0.05M, pH 7.8) and spray reagent (4). A single spot, at a distance of 1.5 cm towards the anode, was obtained for polysaccharide D, but two spots with some tailing were detected in other cases.

Column chromatography, on Sephadex G-100, of polysaccharide D

Polysaccharide D (30 mg) was dissolved in 0.05M ammonium hydrogencarbonate buffer (5 ml, pH 8.17) and was charged on the top of a column (95 x 1.1 cm) of Sephadex G-100. The column was eluted with the same buffer and 5 ml portion was collected in each test tube, monitoring with a differential refractometer. A single peak appeared on the chromatogram, (Figure 2). The pooled fractions (120 ml) was dialyzed for 2 days against distilled water and then lyophilized; yield 28.6 mg, $[\alpha]_D^{23}$ + 65° (c 0.6,

water). A second batch of polysaccharide D was made by following the same procedure. It had $[\alpha]_D^{23}$ + 66° (c 0.5, water).

Estimation of sugars in polysaccharide D

Polysaccharide D (4.56 mg) was mixed with myo-inositol (1.2 mg) and hydrolyzed with 1M sulfuric acid for 20 hours on a boiling-water bath. The hydrolyzate, after the usual treatment, was converted into its alditol acetates[42]. The alditol acetates were analyzed by g.l.c. using column (a) at 190°, (Figure 3). Polysaccharide D was found to contain arabinose (10.8%), galactose (16.4%) and glucose (71.4%).

Methylation analysis of polysaccharide D

Polysaccharide D (5 mg) was dissolved in dry dimethyl sulfoxide (5 ml) and methylated by Hakomori method[45] by treatment with 2M methylsulfinyl sodium (5 ml) and methyl iodide (2 ml). The methylated material, obtained after lyophilization, showed band for O-H stretching vibration in the i.r. spectrum. The product was therefore remethylated by Purdie method[47] using methyl iodide and silver oxide. The permethylated material (3.9 mg) had $[\alpha]_D^{23}$ + 56° (c 0.39, in chloroform) and showed no O-H absorption band in the i.r. spectrum.

The methylated product was hydrolyzed with 85% formic acid (2 ml) for 2 hours at 100°, the acid was removed with co-distillation with water, and the product was heated with 0.5M sulfuric acid at 100° for 18 hours and after the usual treatment, the partially methylated monosaccharides were converted into alditol acetates. The mixture was analyzed by g.l.c. using column (c) at 173°. The results are summarized in Table 5.

Smith[79] degradation of polysaccharide D

Polysaccharide D (7 mg) was treated with 0.1M sodium metaperiodate (8 ml) and kept in the dark for 48 hours at 10° (with the help of a pilot experiment it was found that the rate of consumption of periodate almost ceased after 48 hours). The excess of periodate was destroyed by keeping the reaction mixture with ethylene glycol (2 ml) for 3 hours at room temperature. The mixture was dialyzed against distilled water for 3 days and then concentrated to 2 ml. The concentrated solution was reduced with sodium borohydride (45 mg) overnight at room temperature, acidified with acetic acid and then dialyzed. The dialyzate was concentrated to a small volume and then lyophilized. The product was hydrolyzed with 1M sulfuric acid for 20 hours on a boiling-water bath and the hydrolyzate, after the usual treatment, was converted into alditol acetates. The alditol acetates were analyzed by g.l.c. using column (a) at 190°.

Oxidation of polysaccharide D with chromium trioxide[80,81]

Polysaccharide D (3.7 mg) was mixed with myo-inositol (0.3 mg, internal standard) and then dissolved in formamide (0.7 ml). To this solution were added acetic anhydride (0.8 ml) and pyridine (1.2 ml), and the mixture was stirred for 16 hours at room temperature. The acetylation product was isolated by evaporating the mixture to dryness, and then partitioning between water and chloroform. The chloroform layer was dried (Na_2SO_4) and then evaporated to dryness. The product was dissolved in glacial acetic acid (4 ml) and treated with chromium trioxide (300 mg), with stirring at 50°. Aliquots were removed at 0 hour, 1 hour and 2 hour, diluted with water immediately after removal, and the solutions extracted with chloroform. The extracts were dried (Na_2SO_4) and evaporated to dryness, and the dried materials were deacetylated with sodium methoxide. The products were then hydrolyzed and finally analyzed, as the alditol acetates, by g.l.c. using column (a) at 190°. The results are given in Table 6.

Graded hydrolysis studies on polysaccharide D

Optimum condition for release of highest proportion of oligosaccharides was found out with the help of pilot experiments. In these experiments, polysaccharide D was separately hydrolyzed with different concentrations of formic acid (85%,

40%, 20%, 10%) at 100°. Aliquots at different time interval were taken out, formic acid was removed by codistillation with water under reduced pressure and the hydrolyzates were then examined by paper chromatography. Four hours hydrolysis at 100° with 40% formic acid was found to be the optimum condition for highest release of oligosaccharides.

Polysaccharide D (25 mg) was dissolved in 40% formic acid (50 ml) and hydrolyzed for 4 hours at 100°. Formic acid was codistilled off with water under reduced pressure, oligosaccharides were resolved on Whatman No. 3 MM papers using solvent (F) and the separated sugars were isolated by eluting with water. Two oligosaccharides were obtained in pure state.

Characterization of oligosaccharides

Oligosaccharide - I (1.5 mg)

A small portion (0.5 mg) of this oligomer $[\alpha]_D^{23}$ + 32.2° was hydrolyzed with 0.5M sulfuric acid for 10 hours and after usual treatment, was converted into alditol acetates. G.l.c. analysis of the alditol acetates using column (a) showed the presence of only glucose.

The oligomer (~1 mg) was methylated by Kuhn[50] method. The methylated product was hydrolyzed with 0.5M sulfuric acid for 18 hours and the hydrolyzate was converted into alditol acetates which on g.l.c. analysis in column (c) gave peaks for 2,3,4,6-tetra-O-methylglucose and 2,3,6-tri-O-methylglucose in almost equimolar proportions (1.06 : 1).

Oligosaccharide - II (2.2 mg)

The oligomer had $[\alpha]_D^{23}$ + 60.7°. A part of this oligomer (~0.5 mg) was hydrolyzed as before and the alditol acetates of the hydrolyzate were analyzed by g.l.c. using column (a) which gave only peak for galactose.

The oligomer (~1 mg) was methylated by Kuhn[50] method. The methylated compound was hydrolyzed and the hydrolyzate, after the usual treatment, was converted into alditol acetates which on g.l.c. analysis using column (c) showed 2,3,4,6-tetra-O-methylgalactose and 2,3,6-tri-O-methylgalactose in equimolar proportions (1.03 : 1).