SECTION - D

EXPERIMENTAL

(a) METHOD

(b) ISOLATION OF POLYSACCRIDES AND DETERMINATION OF THE SUGAR PRESENT IN IT.

(c) METHYLATION STUDIES

(d) PERIODATE OXIDATION STUDIES

(e) PARTIAL ACID HYDROLYSIS STUDIES
(a) METHODS

1. EVAPORATION

All evaporation were carried out under reduced pressure at low temperature (bath temperature 45 - 50°C)

2. MELTING POINT

All melting points are uncorrected and they were determined by Gallencamp melting point apparatus.

3. OPTICAL ROTATION

Optical rotations were determined in a Hilger Polarimeter using sodium lamp as the higher source. All the values reported are equilibrium values unless otherwise mentioned.

4. PAPER CHROMATOGRAPHY

Paper chromatography was carried out by descending method on Whatman No. 1 filter paper sheets. Whatman No. 3 MM sheets were used for the quantitative separation of mixture. The following solvent systems (V/V) were used for chromatography.

(A) Butan-1-ol-ethanol-water (4:1:5; upper phase) (105)
(B) Benzene-ethanol-water (167:47:15; upper phase) (106)
(C) Butanone-water azeotrope (107)
(D) Butanone-ethylacetate-water-ammonia (80:20:8:1; upper phase)
Butan - 1 - ol - ethanol - water (31:11:9).

Ethyl acetate - acetic acid - water (9:2:2) (14, 106)

Butan - 1 - ol - pyridine - water (6:4:3) (83, 108)

Ethyl acetate - ethanol - water (7:3:2).

Butanol - acetic acid - water (4:1:5)

Ethylacetate - pyridine - water (2:1:2)

The following spray reagents were used to detect the sugars on chromatogram:

(a) Silver nitrate- Sodium hydroxide reagent (109).

(b) p-anisidine phosphate reagent (110).

(c) 1% aqueous alcoholic sodium metaperiodate solution.

Rf, Rgal, Rglu, Rg values refers to the rate of movement on sugar on paper chromatogram relative to solvent point, D - galactose, D - glucose and 2, 3, 4, 6-tetra - O - methyl - D - glucose, respectively.

5. COLUMN CHROMATOGRAPHY

Following columns were used in column chromatography.

(i) Cellulose Column

(ii) Charcoal Celite column

The following solvents are used as eluants:

(a) Butan-1-ol half saturated with water (III).

(b) Benzenè-ethanol-water (167:47:15 V/V Vapour Phase) (106)

(c) 2.5, 5.0, 7.5, 10.0 and 15.0% aqueous ethanol.
6. **THIN LAYER CHROMATOGRAPHY**

Thin layer chromatography was carried out on silica gel in cyclohexanol - benzene ethanol (10 : 10 : 3, V/V) (112).

7. **ZONE ELECTROPHORESIS**

The electrophoresis was carried out on laboratorium Felzercislk Gyara apparatus, Budapest, Hungary type DE - 201. In the present work the paper electrophoresis was conducted on Whatman No. 1 MM paper (45 X 4 cm.) in borate buffer (0.05M, pH 9.2) at 700 volts and 19.20 mA for seven hours. For oligosaccharides, electrophoresis was maintained at 450 v for 45 minutes only.

8. **INFRARED SPECTROSCOPY**

The infrared spectra were recorded on a Perkin - Elmer model 137-B in the following phases:

(I) in potassium bromide pellets,
(II) in chloroform solution in sodium chloride cells,
(III) in nujol mulls

9. **DEIONISATION**

Deionisation was done with Amberlite IR - 120 (H⁺) and IR - 45 (OH⁻).

10. **DETERMINATION OF METHOXYL VALUE**

The methoxyl content of samples was determined by the Ziesel's method.
11. **PHOTOELECTRO COLORIMETRY**

Absorption photoelectro colorimetry was carried out on Klett summerson photoelectro colorimeter Model 900.3. The absorption was read either at 470 m\(\mu\) or 540 m\(\mu\).

(b) **ISOLATION OF THE POLYSACCHARIDE AND DETERMINATION OF THE SUGARS PRESENT IN IT.**

**ISOLATION OF POLYSACCHARIDE**

The cassia Laevigate seeds (150 g) were cleaved in a low speed grinder when the seedcoat together with the embryo attached to it broke off from the light yellow coloured cotyledons. The cotyledons were separated by winnowing and rejected. The remaining material (90 g) containing the endosperm soaked in water (3 L) over night followed by heating at 100° for 4 hours. The swollen material was then blended with additional quantities of water. The resulting viscous solution (6 L) was filtered through a muslin cloth and centrifuged at 25,000 r.p.m. in a super centrifuge when most of the suspended matter was removed and almost clear solution resulted. Addition of ethanol (14 L) to this solution precipitated the polysaccharide in the form of coarse powder (14 g.). The crude polysaccharide was cream in colour and had sulphated ash 3.5%.

The crude polysaccharide (10 g.) was redissolved in water (5 L) with constant mechanical stirring for 5 hours. To this solution was added saturated barium
hydroxide solution when the polysaccharide precipitated out as its barium complex (114). It was allowed to settle and isolated by decantation followed by filtration. The solid material was stirred with 2 N acetic acid (2 L) for 25 hours when it dissolved partially. After removal of the insoluble fraction by filtration the soluble fraction was isolated by precipitation with ethanol (5 L). The insoluble fraction gave no test for carbohydrate so was rejected. The isolated solid fraction was washed with 70, 80 and 90% ethanol. It was then kept in dry acetone for overnight. The resulting polysaccharide was dried over calcium chloride in vaccum desiccator. The polysaccharide was obtained as a white amorphous powder (yield 10 g.). The sulphated ash was found to be 1.2%.

REDUCTION OF THE ASH BY ION EXCHANGE RESINS

The cation exchange resin, Amberlite IR - 120 and anion exchange resin, Amberlite IR - 45 were taken in a beaker, soaked in water and left overnight. The soaked resins were then packed in two different glass tubes to give the 18 inches columns. The glass tubes were already connected to constant level arrangements and their lower ends were plucked with glass wool. The columns were back washed with water for five minutes at a rate which expanded the resin bed almost doubly, allowed the resin particles to settle and water was drained to a constant level. The cation and anion - exchange resin columns were
regenerated by passing N hydrochloric acid (200 ml.) and dilute ammonia solutions (200 ml.), respectively from the top of the columns at the rate of 5 ml. per minute each. The columns were washed with water until the effluents were almost neutral. The solution of polysaccharide (4 g. in 100 ml.) was then slowly poured into the columns (firstly through cation exchange resin followed by anion exchange resins) and columns were washed with A bed volumes of water. The deionised solution was concentrated to 80 ml. and precipitated with ethanol, filtered, the precipitate washed with acetone and dried.

FRACTIONAL PRECIPITATION

The deionised polysaccharide (10 g.) was stirred for four hours with hot water (1.0 L) and ethanol was added to the solution in stages. After each addition the solution was allowed to stand overnight. Addition of first 200 ml. had no effect, next 150 ml. produced a turbidity which was centrifuged out. Another 100 ml. resulted in slight precipitate (500 mg.) which was removed by filtration. Finally the addition of 150 ml. resulted in copious precipitation of the polysaccharide (8 g.)

PRELIMINARY ANALYSIS OF THE POLYSACCHARIDE

The polysaccharide was in the form of white amorphous powder had \(\left\langle \alpha \right\rangle^D_{25} + 31.2^\circ\) (C, 1.0 H\textsubscript{2}O) and did not reduce Fehlings solution. Nitrogen, Sulphur,
halogens, acetyl group, Uronic acids were absent.

**SULPHATED ASH**: The purified polysaccharide (0.32 g.) was moistened with few drops of sulphuric acid and ignited gently in a Silicon crucible until well carbonised. It was then placed in a muffle furnace at 450° for 5 hours. It was cooled, moistened with sulphuric acid and again ignited at 600° to a constant weight. The sulphated ash of the polysaccharide was 0.12%.

**HOMOGENETITY**: Homogeneity of polysaccharide was confirmed by paper electrophoresis. The polysaccharide did not move at all under various conditions of paper chromatography. It was, however, subjected to paper electrophoresis on strips of (45 X 4 cm.), Whatman No 1 MM filter paper in borate buffer (0.05 M, sodium tetraborate decahydrate), pH 9.2, under a field strength of 700 V for six hours. The compound moved as a single spot. The presence of polysaccharide spot on the paper was located spray reagent (a) and washing with acetone.

**PENTOSAN ESTIMATION**

The method depends upon the treatment of polysaccharide with 12% hydrochloric acid which decomposes pentosans to furfural, the latter being precipitated with phloroglucinol and estimated.

The substance (0.5432 g.) was accurately weighed and transferred to a 500 ml. round bottomed flask fitted with a claisen head, a dropping funnel and a condensor.
Hydrochloric acid (100 ml. 12%) was added to the substance. The flask was heated in a liquid paraffin bath at 170-175° and the distillate was collected at the rate of 30 ml. in 10 minutes. Hydrochloric acid (30 ml. of 12%) was added through the dropping funnel at one time keeping little acid solution in dropping funnel is a liquid seal. The distillation was continued and whenever 30 ml. of distillate was collected in the receiver, same amount of hydrochloric acid was added to the flask. After collecting 240 ml. of distillate it was tested for the presence of furfural by aniline acetate paper. When a negative test was obtained (absence of red colour on aniline acetate filter paper) the distillation was stopped (300 ml.). The distillate was treated with an excess of phlorogluconol solution (prepared by dissolving 3.8014 g of phlorogluconol in 100 ml. hot 12% hydrochloric acid and then making up the volume to 500 ml. by 12% hydrochloric acid and kept for two days). The solution first turned yellow, then green, and finally became almost black when the amorphus dark green precipitate of of furfural phloroglucide began to deposit. The volume of the solution was made upto 400 ml. with 12% hydrochloric acid and left overnight. The precipitate was filtered through weighed sintered glass concible, care being taken to see that the precipitate did not dry during filtration. The precipitate was washed with filtered distilled water
(150 ml.) and dried in oven at 104° for 4 hours. The crucible was weighed and the precipitate was washed with 95% absolute alcohol till the filtrate became colourless. After drying for one hour at 104° the weight of the crucible was recorded and the process was repeated till a constant weight was obtained. From the weight of the phloroglucinide the weight of pentosans was obtained from the standard Krober's table.

Furfural : 3.92%
Pentosans : 6.01%
Pentoses : 7.30%

INFRARED SPECTRUM

The infrared spectrum of the polysaccharide (K Br) showed absorption bands at 817 and 874 cm⁻¹, thus indicating the presence of β-linked D-mannopyranose units.

ACETYLATION OF THE POLYSACCHARIDE

The polysaccharide (0.8 g.) was shaken at room temperature with formamide (50 ml.) for 24 hours. Anhydrous pyridine (12 ml.) was added to the above suspension, followed by dropwise addition of acetic anhydride (10 ml.) for 4 hours, a brown viscous solution resulted. It was kept overnight and the acetylated product was precipitated from it as a greyish white powder by stirring into water. It was washed with water followed by ethanol and finally
with petroleum ether to dry it. The dried acetyl derivative was dissolved in acetone (50 ml.). Small amount of undissolved material was centrifuged out. Pure derivative was then reprecipitated and dried as before, yield 0.72 g. ($\alpha$)\textsubscript{d}$^{25}$ + 25.4 (C, 0.4 acetone).

**REGENERATION OF THE POLYSACCHARIDE FROM THE CORRESPONDING ACETATES**

The acetate derivative of polysaccharide (0.62 g.) was dissolved in acetone (25 ml.) and refluxed with potassium hydroxide (45%, 25 ml.). The lower viscous layer containing alkali and polysaccharide was separated and poured into acidified ethanol. A further precipitation in the same manner with neutral ethanol afforded purified galactomannan which was dried by solvent exchange, yield 0.14 g. ($\alpha$)\textsubscript{D}$^{24}$ + 28.6° (c, 1.0 H\textsubscript{2}O).

**IDENTIFICATION AND ESTIMATION OF SUGARS FROM THE POLYSACCHARIDE**

**ACID HYDROLYSIS**

The polysaccharide (2 g.) was kept overnight in 72% sulphuric acid (115) and was then made up a normal solution with respect to sulphuric acid. The solution was heated 30 hours in boiling water bath, which was followed iodometrically (116) was found to be complete.
RESULTS OF IODOMETRIC TITRATIONS DURING HYDROLYSIS

Iodine solution (5 ml.) of approximately 0.1 N was mixed with 0.1 N Sodium hydroxide solution (10 ml.) and was allowed to stand for 20 minutes. The mixture was acidified with 2 N sulphuric acid (25 ml.). The liberated iodine was titrated with 0.1 N sodium thiosulphate solution.

A portion (1 ml.) of the hydrolysate was pipetted and was neutralised with N sodium hydroxide solution using phenolphthalein as an indicator. The resulting solution was subjected to the same process as above till no change in hypo reading.

TABLE

RATE OF HYDROLYSIS OF THE POLYSACCHARIDE
(HYPO CONSUMED FOR BLANK IS 4.6 ML.)

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Time (h)</th>
<th>Hypoconsumed (ml)</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0</td>
<td>4.6</td>
<td>Hydrolysis with 72%</td>
</tr>
<tr>
<td>2.</td>
<td>10</td>
<td>4.0</td>
<td>Sulphuric acid at room temperature</td>
</tr>
<tr>
<td>3.</td>
<td>15</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>20</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>25</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>30</td>
<td>2.1</td>
<td>Further hydrolysis with N sulphuric acid at 100°</td>
</tr>
<tr>
<td>7.</td>
<td>35</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>40</td>
<td>2.1</td>
<td></td>
</tr>
</tbody>
</table>

The hydrolysed solution was neutralised with barium carbonate and kept overnight. Barium sulphate and
Unreacted barium carbonate were removed from the solution by filtration and the residue was washed with hot water (50 - 60°) several times. The combined filtrate and washings were deionised by Amberlite IR - 120 (H⁺) and Amberlite IR - 45 (OH⁻) ion exchange resins and concentrated to a thin syrup. Paper chromatographic examination using solvent (A) and spray reagent (a) and (b) showed the presence of corresponding spots of D-galactose (faint), and D-mannose (strong).

CHARACTERISTICS OF THE SUGAR

Separation of the sugars

The sugar syrup obtained by the hydrolysis of the polysaccharide was resolved into pure components on a cellulose column with Butan-1-ol half saturated with water (eluant).

The cellulose column was separated by modified slurry method of packing. An ordinary glass column (2.5 cm. diameter) having a stopcock at the lower end was used for making the column. The glass wool was placed at the bottom of the tube. The Whatman standard grade cellulose powder was added to bulanol half saturated with water in a blender and a medium slurry was made. The suspension was allowed to run out from the stopper of the tube. As the solvent ran out of the tube, cellulose powder settled gradually giving a uniform bed. During the
process the column was not allowed to run dry at any stage, otherwise air bubbles would be formed and the packing procedure had to be repeated. The chromatographic tube was kept nearly full of slurry at all the time till the column was packed to the desired height (23 cms.). The surface of the cellulose was kept flat and horizontal in order to avoid distortion of the zones of sugar, and it was covered by a thick filter paper piece (Whatman No. 3) to prevent the surface from being disturbed. Before use the column was washed with butanol half saturated with water which was supplied from a constant head reservoirs.

Methylred dye was used to check the uniformity of the packing. The solvent of the column was allowed to drain to the top of the cellulose and the methyl red dye (in solvent) was added from the pipette over the top of the cellulose column. The dye was allowed to soak into the cellulose and washed out with the solvent. The horizontal band moving of the dye in the entire length of the column proved the uniform packing of the column.

The first washings were brownish and subsequent washings were lighter in colour. Washings were continued till the was liquid coming out from the column was perfectly colourless.

The hydrolysate syrup of the polysaccharide was diluted with a little water and was introduced on the column and allowed to soak into the cellulose. The column
was then eluted with solvent. The eluate was collected in approximately 10 ml fraction. Each fraction was examined by paper chromatography on Whatman No. 1 filter paper sheets using solvent \( A \), appropriate fractions of the individual sugars as shown in the table, were combined and evaporated to dryness to obtain pure specimens of sugars.

**TABLE**

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Fraction No(s)</th>
<th>Sugar Identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>1 - 20</td>
<td>No sugar</td>
</tr>
<tr>
<td>2.</td>
<td>21</td>
<td>Faint spot of mannose</td>
</tr>
<tr>
<td>3.</td>
<td>22 - 48</td>
<td>D - Mannose only</td>
</tr>
<tr>
<td>4.</td>
<td>49 - 58</td>
<td>Mixture of Mannose and Cialactose</td>
</tr>
<tr>
<td>5.</td>
<td>59 - 100</td>
<td>D - Galactose only</td>
</tr>
</tbody>
</table>

**Fraction Nos. 22-48**

It gave a syrup on concentration which had Rf: 0.11 and 0.21 in solvent (A) and (I) respectively. This syrup was purified with alcohol - water mixture and decolourised by active charcoal. On concentration and crystallisation from ethanol gave D - mannose (m.p. and mixed m.p. 130-131°; Lit. m.p. 132°); \( \alpha(\text{D})^{32} + 13.8° \) (in water) (Lit. \( \alpha(\text{D})^{25} + 14.1° \)). Both the values are in good agreement with the reported values for D - mannose. For further confirmation the sugar syrup (0.110g.) was refluxed
with methanolic solution of p-nitroaniline (0.1020 g.) containing a trace of hydrochloric acid furnished a crystalline p-nitro-N-phenyl-D-mannosyl amine (117). After crystallisation from methanol the m.p. was found to be 212-214° remained undepressed on admixture with an authentic samples, \( \alpha \)\(^{35}\) \(_D\) 328° C, 0.6 dry pyridine

**Fraction Nos. 59-100**

It gave a syrup on concentration which Rad Rf: 0.07 and 0.16 in solvent (A) and (I), respectively. On purification and recrystallisation with ethanol it had m.p. and mixed m.p. 163-164° (lit. m.p. 167°, \( \alpha \)\(^{32}\) \(_D\) + 80.2° in water). Both the values are in good agreement with the reported values for D-galactose. For further confirmation p-nitro-N-phenyl-D-galactoxylamine derivative was prepared from the crystalline sugar in the same way as has been described in the case of mannose. It had m.p. and mixed m.p. 214 - 216°, \( \alpha \)\(^{35}\) \(_D\) 230° (C1 0.6 in dry pyridine).

**QUANTITATIVE ESTIMATION OF THE SUGARS [62]**

The polysaccharide was hydrolysed with N sulphuric acid in sealed tube at 100° for 30 hours and the hydrolysate was processed as usual. The resulting sugar mixture was separated on Whatman No 1 chromatographic sheets using solvent (I). The corresponding strips of the
sugars were cut with the help of guide spots and were eluted by Dent's method (118). The eluted sugars were estimated by periodate oxidation method (62) and phenol sulphuric acid method (119). The molar ratio of D-galactose and D-mannose was found to be 1:1.5 (2:3)

(C) METHYLATION STUDIES

Polysaccharide (15 g.) was dissolved in water (100 ml.) and methylated (67) by dropwise addition of dimethyl sulphate (120 ml.) and 45% sodium hydroxide solution (200 ml.) with vigorous stirring in an atmosphere of nitrogen. The addition was carried out over a period of 4 hours keeping the solution always alkaline. For the first few hours the reaction was carried out at low temperature (0-5°) and after that the bath temperature was raised 15-20°. The mixture is then stirred for 12 hours. The whole process was repeated with a fresh lot of sodium hydroxide 45%, 200ml.) and dimethyl sulphate (120 ml.) following the same conditions. The solution gave a negative test with Fehlings solution showing the complete formation of methyl glycosides. The reaction mixture was stirred for 12 hours and heated 80-85°c for two hrs. to decompose excess of dimethyl sulphate. The solution was cooled in an ice bath and neutralised with cold 15N sulphuric acid to a pH 7.0. The sodium sulphate formed was filtered out and the filtrate was evaporated upto 100ml. The partially methylated polysaccharide was
Figure 22

Transmittance (%) vs. Wavelength (microns)

CM⁻¹

0 1000 900 800 700

4000 3000 2000 1500

0 15 14 13 12 11 10 9 8 7 6 5 4 3

60 80 100

WAVEL ENGT (MICRONS)
extracted with chloroform in a liquid-liquid extractor. The chloroform extract was dried over anhydrous sodium sulphate and evaporated to a light yellow coloured syrup and finally to a solid mass and dried in vacum, yield 8.6 g. it had -OCH₃, 36.0%. The infrared spectrum of the polysaccharide in chloroform solution showed a prominent hydroxyl peak in the region 3400-3600 cm⁻¹ indicating lack of complete methylation (fig. 22).

The above methylated product (8.2 g.) was dissolved in tetrahydrofuran {71} (200 ml.) and the powder of sodium hydroxide (75 g.) was added, stirred till all the powder was dissolved. The addition of dimethylsulphate (90 ml.) was carried out over a period of 4 hours and stirred for 20 hours in a water bath at 20 to 25°C. Enough water was added to bring the solids into the solution. The resultant solution was refluxed for one hour at 60°. The tetrahydrofuran was removed by aeration for 15 hours and the solution was neutralised with 15N sulphuric acid in a ice bath to a neutral pH. The sodium sulphate was removed by filtration and the filtrate was evaporated to 50 ml. The partially methylated polysaccharide was extracted with chloroform. The extract was dried over anhydrous sodium sulphate and evaporated to yellow glassy solid yield 8.0g. -OCH₃ 40.0% . The solid obtained showed -OH absorbance at 3500-3600 cm⁻¹ in IR spectrum (fig. 23).

The partially methylated product so obtained was
FIG-23
further methylated by Purdie's method (72). The substance (8.0 g) was dissolved in A.R. acetone (75 ml.) containing methyl iodide (60 ml.) and freshly prepared silver oxide (25 g.) was added in several batches over a period of 6 hours, with continuous stirring. It was refluxed for 12 hours in a water bath at 50-60°C. The methyl iodide was distilled off at 40-42°C and the resultant solution was filtered. The filtrate was dried over anhydrous sodium sulphate. The silver oxide was soxhletated with chloroform and the extract was dried over anhydrous sodium sulphate. The filtrate and chloroform extract was evaporated together to a yellow glassy solid. This methylation procedure by Purdie's method was repeated twice, the methylated polysaccharide (6.8 g.) obtained was tested and the completion of the methylation. It had \(-\text{OCH}_3, 43.0\%\), \(\left\langle \alpha \right\rangle_{\text{D}}^{20} + 38.4\) (Chloroform) and showed no hydroxyl absorption in the infrared spectrum (fig. 24). Further methylation by this procedure did not increase the methoxyl content.

**FRACTIONATION OF METHYLATED POLYSACCHARIDE**

Methylated polysaccharide (8.0 g.) was treated with a mixture of purified light petroleum ether (b.p. 40-60°C) and chloroform, the amount of the latter solvent being increased in stages. The mixture was refluxed gently in a water bath, 2 hours, for each extraction. The insoluble material was then allowed to settle, and the clear solution
was decanted. The solvent was removed under reduced pressure and the residue was dried under high vacuum to constant weight. The results are summarised in table.

**TABLE**

**FRACTIONATION OF METHYLATED POLYSACCHARIDE**

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Solvent Peř. ether/Chloroform</th>
<th>yield (g.)</th>
<th>-OCH$_3$ (%)</th>
<th>$[\alpha]_{D}^{28}$ Chloroform</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>100 0</td>
<td>1.2102</td>
<td>38.9</td>
<td>+12.8</td>
</tr>
<tr>
<td>2.</td>
<td>90 10</td>
<td>3.0112</td>
<td>42.6</td>
<td>+40.8</td>
</tr>
<tr>
<td>3.</td>
<td>85 15</td>
<td>2.4202</td>
<td>43.5</td>
<td>+40.1</td>
</tr>
<tr>
<td>4.</td>
<td>80 20</td>
<td>0.0716</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5.</td>
<td>70 30</td>
<td>0.0614</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The composition 2 and 3 which were analytically similar and together formed the major component (5.4314 g) were obtained and further study was carried out only on this material. The infrared spectrum of this fraction showed no hydroxyl peak in the region 3400 - 3600 cm$^{-1}$.

**HYDROLYSIS OF THE METHYLATED POLYSACCHARIDE**

The methylated polysaccharide (fraction 2 and 3, 3.0 g.) was dissolved in 10 ml. 72% sulphuric acid in a round bottom flask, externally cooled with ice water and the solution was kept for one hour at room temperature. It was diluted to 8% of sulphuric acid with water (100 ml.)
and heated for 4 hours on a boiling water bath. It was then neutralised with barium carbonate. The solids were separated by filtration and carefully washed with water and chloroform. The precipitate of barium sulphate and carbonate was refluxed with methanol (20 hours). The filterate washings of water and chloroform methanol extract were concentrated together to a thin syrup, yield 2.8 g.

The paper chromatographic analysis of the mixture using solvent (B) and (C) and spray reagent (b) showed following spots as given in table

**SUGARS PRODUCED ON HYDROLYSIS OF METHYLATED POLYSACCHARIDE**

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Rf. Solvent (C)</th>
<th>Probable Methylated Sugars</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0.23</td>
<td>2,3-di-0-methyl-D-mannose</td>
</tr>
<tr>
<td>2.</td>
<td>0.50</td>
<td>2,3,6-tri-O-methyl-D-mannose</td>
</tr>
<tr>
<td>3.</td>
<td>0.72</td>
<td>2,3,4,6-tetra-O-methyl-D-galactose.</td>
</tr>
</tbody>
</table>

**SEPARATION AND PURIFICATION OF METHYLATED SUGARS**

The hydrolysate was resolved into its pure components by paper chromatography using 3 MM Whatman sheets and solvent (B) as irrigant. The corresponding
zones of the components were cut out with the help of guide spots, eluted with water separately and evaporated to the thin syrups under high vacuum. Each component was purified with chloroform or methanol decolourised with active charcoal and then evaporated to a thin syrup, dried under high vacuum to constant weight. The amount of various sugars obtained is shown as follows:

- fraction I --- 812 mg.
- fraction II --- 422 mg.
- fraction III --- 878 mg.

IDENTIFICATION OF THE METHYLATED SUGARS

Fraction I : 2,3-di-O-methyl-D-mannose

On paper chromatogram, it moved a single spot in solvent (B) and (C) had Rf: 0.23 in solvent (C). The methoxyl contents was found to be 29.2% (calculated for dimethyl hexose - OCH₃, 29.8%). After drying syrup had \[ \{\alpha\}_{D}^{28} - 15.2^\circ \text{(c, 0.6 H}_2\text{O)} \text{ and } \{\alpha\}_{D}^{28D} + 6.3^\circ \text{(c, 0.5 MeOH)}; \{\alpha\}_{D}^{28D} - 17 \text{(H}_2\text{O)} \text{ and } + 6^\circ \text{(MeOH)}\), -15.8° (H₂O) and + 6° (Me OH), -16° (H₂O) \{37\}, -14° \{14\}, -15.5° and +6.1° (methanol) \{18\}, -15° and +5.7° (methanol) and -16.5°. Demethylation of a position with 48% hydrobromic acid gave only one hexose component which chromatographically corresponds to mannose along with other partial demethylated products.

The methylated product (25 mg.) was dissolved in dry pyridine (5 ml.) treated with p-nitrobenzyl chloride (120 mg.) for 40 minutes at 60-70° and left over
night at room temperature. A saturated solution of sodium bicarbonate was added dropwise to the reaction mixture until no further effervescence occurred. Water was added and the product was extracted with chloroform thrice. After drying the solution with anhydrous sodium sulphate, the solvent was evaporated and the solid obtained was recrystalline with methanol. The 2,3-di-O-methyl-D-mannose, 1,4,6-tri-p-nitrobenzoate had m.p. 191-193° (Lit m.p. 192-194°, 192-193° and 191-193°)

**Fraction II 2.3.6-tri-O-methyl-D-mannose**

On paper chromatogram it moved a single elongated spot in solvent (B) and (C), had Rf: 0.50 in solvent (C). The methoxyl contents was found 42.6% (calculated for tri-O-methyl-mannose, OCH₃, 41.9% After drying the syrup had (α)₂₅D -10° (c, 1.0 H₂O) (Lit. value: (α)D -6.5°, -9°, -7.6°; -10° (41).

Demethylation of a position with 48% hydrobromic acid gave mannose and traces of galactose on paper chromatogram. Paper chromatography, specific rotation and demethylation data suggested that this component corresponding to 2,3,6-tri-O-methyl-D-mannose together with a tri-O-methyl-D-Galactose. Attempts were made to separate the two methylated sugars by paper chromatography using different solvents did not succeed, but it chromatographic behaviour showed that it may be 2,3,6-tri-O-methyl-D-galactose.

The syrup (25 mg.) was refluxed with aniline (11mg)
and absolute ethanol (4 ml.) for 8 hours under anhydrous conditions. The resulting solution was evaporated at 40° under high vaccum and the solid obtained was recrystallised with ethyl acetate ether mixture affording N-phenyl-2,3,6-tri-0-methyl-D-mannoxyll amine, m.p. 131°; (lit. m.p. 127-128° {120}, 127-129° {6}, and 130-132° {121}).

The syrup (40 mg.) was oxidised with bromine water (20 ml.) and kept at room temperature for 4 days. The bromine was removed by passing a current of air and the solution was neutralised with silver carbonate and filtered. Hydrogen sulphide was passed to precipitate silver sulphide, the clear solution was evaporated to a syrup. The syrupy product was lactonised by being heated at 170°/0.1mm pressure for half an hour. The product on recrystallisation with petroleum-ether (60-80°) furnished needle shaped colourless crystals of 2,3,6-tri-0-methyl-D-manno-Y-lactone, m.p. 84°.

Fraction III : 2,3,4,6-tetra-0-methyl-D-galactose

On paper chromatography, it moved as a single spot in solvent (C) and had Rf: 0.71. The methoxyl contents was found to be 52.1% (calculated for tetra-0-methyl-D-galactose, -OCH₃ 52.5%). After drying the syrup had [α]D³² + 112° (c,0.5 H₂O) (Lit. value + 110° {18}, + 118° {41} and +112° {37}). Demethylation of a postion with hydrobromic acid(48%, 1 ml.) gave only hexose components
which chromatographically corresponds to D galactose together with partially demethylated sugars.

The anilide was prepared by refluxing the syrup (25 mg.) with freshly distilled aniline (10 mg.) with absolute alcohol (3 ml.) for 8 hours. The reaction was carried under completely anhydrous condition. The solution on slow evaporation in vaccum desiccator, furnished needle shaped crystals. On recrystallisation from ethyl acetate produced the crystals of N-phenyl - 2,3,4,6-tetra-O-methyl-D- galcatosylamine m.p. - 190° (Lit. m.p. 195° [11], 192-193° [4], 194° [18°] and 190° [37].

QUANTITATIVE ESTIMATION OF THE METHYLATED SUGARS

The sugar mixture (about 100 mg.) was seperated by paper chromatography using solvent (C). The strips corresponding to tetra,tri,di,-0-methyl-sugars detected by the help of guide spots, were eluted seperately with 5ml. of water each in a round bottomed flask having ground glass stopper. In each sugar solution 0.1N solution of iodine (1ml.) and then a solution of 2 ml. containing 0.2 M solution hydrogen carbonate and 0.2 M sodium carbonate (pH 10.6) was pipetted and the flask were stoppered. In order to prevent loss of iodine due to evaporation the stoppers were moistened witha little potassium iodide solution (10%). A blank determination was carried out
similarly. After two hours the reaction mixture was
diluted to 25 ml. with water and then acidified by
carefully running 2N sulphuric acid (5 ml.) down the side
of the flask and thus avoiding vigorous evolution of carbon
dioxide. After acidification, the flasks were stoppered
and pending titrations of the liberated iodine was done
with 0.1N sodium thiosulphate solution, using starch as
indicator. A bank estimation was also performed in the
similar way.

The ratio of the methylated sugars determined as a
mean of duplicate experiments is given in table.

**TABLE**

**MOLAR RATIO OF THE METHYLATED SUGARS**

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Methylated Sugar</th>
<th>Molar Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>2, 3, di-O-methyl-D-mannose</td>
<td>2.03</td>
</tr>
<tr>
<td>2.</td>
<td>2, 3,6-tri-O-methyl-D-mannose</td>
<td>1.00</td>
</tr>
<tr>
<td>3.</td>
<td>2,3,4,6-tetra-O-methyl-D-galactose</td>
<td>2.010</td>
</tr>
</tbody>
</table>
PERIODATE OXIDATION STUDIES

The purified polysaccharide (0.3012 g) was dissolved in water (50 ml.) over night and the solution was cooled to 0°C. The sodium metaperiodate 0.12 M (30 ml) was added and volume was made upto 100 ml. The mixture was kept in a refrigerator at 5° to 10°C. A blank was also set up in the similar way. Aliquots (5 ml) were pipetted out at different time intervals to determine the amounts of periodate consumed and formic acid liberated.

(a) Determination of the Amount of Formic Acid Liberated [122].

The aliquots (5 ml) was taken in a flask containing ethylene glycol (2 ml) to destroy the excess of periodate. Potassium iodide (1 g.) and sodium thiosulphate solutions (0.02 N, 5 ml) were added and the resulting solution was titrated against standard iodine solution using starch as indicator (Fig. 25).

(b) Determination of Periodate Consumed by the Method of Fleury and Lange [123]

The aliquots (5 ml) was taken in a flask containing sodium arsenite solution (0.1 N, 10 ml), saturated sodium bicarbonate solution (10 ml) and potassium iodide (1 g). The flask was kept in dark for 15 minutes and then excess of arsenite was titrated with standard standard iodine solution using starch as indicator (Fig. 26). The periodate consumed and formic acid
FIG. 25 PERIODATE OXIDATION OF POLYSACCHARIDE
TIME IN HOURS

FIG. 26 PERIODATE OXIDATION OF POLYSACCHARIDE
liberated per mole of anhydrohexose unit at different intervals of time are given in the table.

<table>
<thead>
<tr>
<th>Hours</th>
<th>10</th>
<th>30</th>
<th>60</th>
<th>90</th>
<th>120</th>
<th>150</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periodate Consumed (Moles/mole of anhydrohexose unit)</td>
<td>0.237</td>
<td>0.59</td>
<td>0.89</td>
<td>1.19</td>
<td>1.29</td>
<td>1.29</td>
</tr>
<tr>
<td>Formic Acid Liberated (Moles/mole)</td>
<td>0.14</td>
<td>0.214</td>
<td>0.24</td>
<td>0.33</td>
<td>0.35</td>
<td>0.35</td>
</tr>
</tbody>
</table>

**PERIODATE DEGRADATION OF THE GALACTOMANNAN**

The polysaccharide (1.8 g) was oxidised with sodium metaperiodate as mentioned before. The excess of periodate was destroyed by ethylene glycol, the solution was dialysed in running water for 5 days and concentrated to 300 ml. To this solution was added sodium borohydride (1 g.) and the solution was stirred well for 4 hours and left at room temperature for 20 hours more. Excess reductant was decomposed by the addition of dilute acetic acid and the solution was dialysed against running water for 25 hours. The dialysed solution was concentrated to a syrup and was hydrolysed with 1 N sulphuric acid (100 ml.) for 12 hours at 100°C. The hydrolysate was neutralised with barium carbonate, filtered, deionised by passage through column
of Amberlite ion exchange resins IR-120 (H⁺) and IR-45 (OH⁻) and concentrated to a syrup. The chromatographic examination in solvent (A) revealed the presence of glycerol, erythritol and traces of mannose. The alcohols gave very prominent spots if the developed chromatogram passed through silver nitrate and was sprayed with 1% aq. alcoholic sodium metaperiodate before the final sodium hydroxide spray.

The concentrate 0.5 g. was resolved into its components on Whatman No.3 MM filter paper sheets using solvent (A). The strips corresponding to each substance were cut out, eluted with water and resulting solution was evaporated to obtain the respective syrups.

**Fraction 1, Glycerol**

The syrup obtained was purified by dissolving it in ethanol (5 ml) filtered, concentrated to a syrup and dried under high vacuum at 60-75°C, yield 85 mg. On paper chromatogram it moved as a single spot and parallel to an authentic specimen of glycerol.

The residue was dissolved in pyridine (5 ml) and p-nitrobenzoyl chloride (2.0 g) was added. The mixture was heated for 45 minutes at 70-75°C and then the solution was poured into ice cold saturated sodium bicarbonate solution. The insoluble product formed was separated by filtration, washed with water, dissolved in hot acetone (2 ml) and left over night. The crude crystals obtained were recrystallised
two times from acetone to give glycerol tri-p-nitrobenzoate, m.p. and mixed m.p. 185-186°C (lit. [4] m.p. 191°C, 185-186°C [18]).

**Fraction 2, Erythritol**

The eluate, containing erythritol fraction, was concentrated to a syrup and purified by treatment of its aq. solution with charcoal. The clear filtrate was concentrated to a thick syrup and dissolved in minimum amount of ethanol and cooled. Upon cooling erythritol crystallised out from the solution. The crystals were filtered and on recrystallisation from ethanol had m.p. and mixed m.p. 117-118°C (lit. m.p. 121°C [18], 117-119°C [121]; 120-121°C [124]).

To an ice cold solution of the erythritol in anhydrous pyridine (2 ml.), p-toluene sulphonyl chloride (4 g.) was added. The reaction mixture was allowed to stand for 24 hours at room temperature and it was poured into water (30 ml). Upon cooling, the derivative crystallised and the crystals were washed with water followed by ethanol and were dried in air. Recrystallised from acetone-ethanol gave tetra-O-tosyl-erythritol, m.p. and mixed m.p. 165°C (Lit. m.p. 164°C [4]; 164-165°C [18,40]; 165-166°C [124]).

**Fraction 3**

It was obtained in traces and was found moving parallel to D-mannose on paper chromatogram, had Rf: 0.11 and 0.32
in solvent (A) and (J), respectively. Further characterisation of the sugar could not be done being its very less in quantity.

QUANTITATIVE ESTIMATION OF GLYCEROL AND ERYTHRITOL IN THE PERIODATE DEGRADATION PRODUCT

The mixture was separated on a Whatman No.1 MM paper sheet and strips containing pure components were cut out with the half of guide strips and eluted with water. The ratio of glycerol and erythritol was determined by periodate-chromotropic acid method. The molar ratio of the component is given in table.

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Component</th>
<th>Molar Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glycerol</td>
<td>1.94</td>
</tr>
<tr>
<td>2</td>
<td>Erythritol</td>
<td>3.00</td>
</tr>
</tbody>
</table>
PARTIAL ACID HYDROLYSIS STUDIES

The polysaccharide was heated on water both (95-100°C) for two hours with 0.5 N sulphuric acid (800 ml.). The resultant solution was cooled to 0 to 5°C in ice bath. The unhydrolysed polysaccharide obtained on filtration, was hydrolysed again with 0.25 N sulphuric acid (500 ml.). Finally unhydrolysed polysaccharide was rejected after filtration of the solution. Both the hydrolysate were combined together, neutralised with barium carbonate in an ice bath and filtered. The filtrate was evaporated to a small volume (10 ml.). Paper chromatographic examination of the mixture using solvent (I) and spray reagent (b) showed the presence of galactose and mannose together with number of oligosaccharides.

Preparation of Charcoal Celite Column for the Separation of Oligosaccharides

The merk charcoal was heated with 6 N hydrochloric acid for 10 hours, on a boiling water bath to hydrolyse unburnt wood material present. The charcoal powder was thoroughly washed with water till the washings were neutral to pH paper and dried at 100°C. The celite was also treated with 6 N hydrochloric acid and worked up as above. Equal amounts (by weight) of the charcoal celite were mixed together, made into a slurry with water and poured into a chromatographic tube (one inch diam.) at the lower end
of which a pad of glass-wool was packed. The slurry was allowed to settle upto 23 cm under gravity and the excess of water was run down slowly through the stopper of the chromatographic tube, the column was washed with five litre of distilled water.

The partially hydrolysate of the polysaccharide (8 g.) was added to the top of the column slowly and allowed to soak on the top of it. Water (5 lit.) was passed through the column under 8 Lts./sq. inch pressure to remove the monosaccharides. The eluate was evaporated into a thin syrup, which on paper chromatographic examination using solvent (I) and spray reagent (b) showed the presence of only monosaccharides, i.e. galactose and mannose.

The column was then eluted successively with 5 litres each of 2.5%, 5%, 7.5%, 10% of aq. ethanol. 250 ml. fraction were collected from each gradient elutions and examined by paper chromatography after evaporation to a syrup. All the fraction of the gradient elution were found to contain mixtures of the oligosaccharides. The following table shows various eluants and the fraction present in them.

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>2.5% Aqueous Alcohol</th>
<th>5.0% Aqueous Alcohol</th>
<th>7.5% Alcohol (aq.sol.)</th>
<th>10% Alcohol (aq.sol.)</th>
<th>15% Alcohol (aq.sol.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2.</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3.</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4.</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>5.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

(+++), (++), and (+) show proportion as indicated by the intensity of spot on paper chromatogram. (-) Absence of the fraction as indicated by the paper chromatogram.
FURTHER SEPARATION OF OLIGOSACCHARIDES
BY PAPER CHROMATOGRAPHY

All the gradient fractions were mixed together to get a mixture of the oligosaccharides. The resultant mixture of oligosaccharides was further resolved into its pure components on the Whatman No.3 MM filter paper in solvent (I). The different oligosaccharides zones were cut out with the help of guide spots, eluted with water, evaporated to a glassy syrup and found to be chromatographically homogeneous. Thus two disaccharide and two trisaccharides were obtained. The oligosaccharides were purified by dissolving in aqueous methanol, decolourised by treatment with active charcoal and dried under high vacuum.

The disaccharides and trisaccharides were characterised in the following way.

Fraction 1
Disaccharide 1

It was identified as 4-0-B-D-mannopyranosyl-D-mannose. The syrup (7 mg) was hydrolysed with N sulphuric acid (2 ml). On chromatographic examination of the resulting syrup obtained after usual treatments, only mannose was found to be present. The oligosaccharide was dissolved in minimum of aqueous methanol, filtered and concentrated to white amorphous solid. It was decolourised with charcoal. The oligosaccharide was chromatographically pure as it gave
only one spot on paper chromatogram using solvent 'I', F; yield 0.332 g. It had Rgal: 0.62 in solvent 'F' and \[\alpha\]_D \text{ -9° (C, 1.2 H}_2\text{O)} (Lit. value -9° [34,36], -7.5° [21]).

The phenyl hydrazone of the oligosaccharide was prepared by refluxing a solution, containing syrup (25 ml) in H\text{2}O (2 ml) and CH\text{3}COOH (0.2 ml) with phenylhydrazine (0.5 ml) at 90-100° for 1 hr. The precipitate obtained was washed with water, benzene and dried under reduced pressure at 60°C. The resulting substance obtained was recrystallised from benzene alcohol mixture; m.p. 205-206°C (Lit. [125] m.p. 203-206°).

The periodate oxidised product was neutralised with barium hydroxide. The precipitate of barium periodate and iodate were removed by filtration. The filtrate (100 ml.) was reduced by sodium borohydride (60 mg) at room temperature for 3 hours. The residue was neutralised with acetic acid evaporated to dryness and hydrolysed with N-sulphuric acid for 7 hours on water bath (100°C). The hydrolysate was neutralised with barium carbonate, filtered and concentrated to a syrup. On chromatographic examination of syrup using solvent 'A', spots corresponding to glycerol (Faint) and erythritol were detected.

**Fraction 2**

**Disaccharide 2**

It was characterised as 6-O-D-galactopyranosyl-D-mannose syrup (yield: 0.142 g.) having Rgal: 0.56 in solvent 'F' was found to be chromatographically pure. The amorphous
solid was dissolved in a minimum of water and methanol in excess was added. After heating and filtering, equal volume of butanol-1 was added. The solution was evaporated on a steam bath to slight turbidity. On cooling sugar crystals in the form of cubes are obtained. It was recrystallised by repeating the above method m.p. was 202° and $[\alpha]_D^{3^\circ} + 124^\circ$ (C, 0.6 H$_2$O); (lit. m.p. 201 - 201.5° [29]; 202° [36]; 201° [21] and 203° [36]; $[\alpha]_D^{120^\circ} + 120^\circ$ [36], 120.9 - 124.6° [41] and 122.5 - 122.1° [36].

The degree of polymerisation as determined by Timells method [99] was 1.82 which indicated it to be a disaccharide.

Acid hydrolysis of the disaccharide with N sulphuric acid afforded D-galactose and D-mannose in equimolar proportions as estimated by phenol sulphuric acid method. Reduction of disaccharide (15 mg) with sodium borohydride followed by hydrolysis gave galactose only on examination by paper chromatography indicating the reducing end to be mannose.

The disaccharide (50 mg) was dissolved in water (2 ml), added to it were phenyl hydrazine hydrochloride (80 mg) and sodium acetate hydrate (120 mg). The phenyl osazone was prepared as described in the case of disaccharide 1. The osazone after recrystallization with 50% aqueous ethanol, had m.p. 174-175°; (Lit. 29 m.p. 175-176°). This osazone in admixture with an authentic specimen of melibiosazone gave no depression in the melting point.
Fig. 27. Periodate oxidation of disaccharide - 1
FIG. 28. PERIODATE OXIDATION OF DISACCHARIDE -1
The oxidation of disaccharide (50 mg.) with 0.2 sodium metaperiodate (15 ml), made up to 100 ml with water at 5 to 9°C, was followed by determining the formic acid liberated and uptake of periodate at various intervals of time the corresponding values are shown in table and figures.\(^{29,30}\)

The degree of polymerisation was found by Timell's method \([99]\). Klett reading of the oligosaccharide hydrolysate before reduction was 180 and after reduction was 65. Thus,

\[
\text{Reduction quotient } Q = \frac{180}{65} = 2.769
\]

and D.P. \[\frac{2.769}{2.769 - 1} = \frac{2.769}{1.769} \approx 1.565\]

The oxidation of mannobiose (50 mg) with 0.2 M sodium metaperiodate (15 ml), made up to 100 ml with water at 5 to 9°C, was followed by determining the formic acid liberated and uptake of periodate at various intervals of time. The corresponding values are shown in table (Fig.27, 28).

<table>
<thead>
<tr>
<th>Table</th>
<th>Periodate Oxidation of the Disaccharide-1 at 5 to 9°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time in Hours</td>
<td>10</td>
</tr>
<tr>
<td>Periodate Consumption (in moles/mole of disaccharide)</td>
<td>2.0</td>
</tr>
<tr>
<td>Formic Acid Liberated (in moles/mole of disaccharide)</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Fig. 29. Periodate Oxidation of Disaccharide - 2
FIG. 30. PERIODATE OXIDATION OF DISACCHARIDE -2
Table
Periodate Oxidation of the Disaccharide-2

<table>
<thead>
<tr>
<th>Time (Hours)</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>55</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periodate Consumed (in moles/mole of disaccharide)</td>
<td>3.8</td>
<td>4.9</td>
<td>5.6</td>
<td>6.0</td>
<td>6.2</td>
<td>6.2</td>
</tr>
<tr>
<td>Formic Acid Liberated (in moles/mole of disaccharide)</td>
<td>2.6</td>
<td>3.8</td>
<td>4.5</td>
<td>4.9</td>
<td>4.92</td>
<td>4.92</td>
</tr>
</tbody>
</table>

**Fraction 3**

Trisaccharide 'A', \(4\text{-}0\text{-}(6\text{-}0\text{-}\beta\text{-}D\text{-}galactopyranosyl-B\text{-}D\text{-}mannopyranosyl)} - \beta\text{-}D\text{-}mannose

The fraction (160 mg) having Rgal 0.34 in solvent (F) was chromatographically pure. The solid was dissolved in minimum amount of 50% ethanol by heating, large volume of hot absolute ethanol was added to it and it was cooled slowly. The partially crystallised oligosaccharide was obtained which filtered and when recrystallised from 85% aq. ethanol, yielded the sugar in the form of prisms. After one more recrystallisation from the same solvent, m.p. was 229°C and \([\alpha]_D^{28} + 95.2 \quad 98.1°\text{C in hours (C, 1.0 H}_2\text{O); (Lit. [126] m.p. 228-229° and } [\alpha]_D^{25} + 93.3 - 98.4°).\]

The degree of polymerisation was 2.87 as determined by Timells method indicating that the oligosaccharide is a trisaccharide. Acid hydrolysis of the trisaccharide (10 mg) with N sulphuric acid solution produced D-galactose and
FIG. 31. PERIODATE OXIDATION OF TRISACCHARIDE
FIG. 32 PERIODATE OXIDATION OF TRISACCHARIDE

FORMIC ACID LIBERATED (moles/mole)

TIME IN HOURS
D-mannose in the molar ratio of 1:1.98 as determined by phenol sulphuric acid method. Reduction of the trisaccharide with sodium borohydride, followed by hydrolysis produced galactose and mannose in approximately equal ratio as shown by the intensity of spots on paper chromatograms. It showed that out of two, one mannose unit of the trisaccharide occupied reducing end position.

The sugar (15 mg) was refluxed with 0.1 N sulphuric acid (2 ml.) for 4 hours at 100°C. The reaction mixture was cooled, neutralised with barium carbonate, filtered and the filtrate was concentrated to syrup. Paper chromatographic examination of the hydrolysate using the solvent (F) and spray reagent (b) revealed the presence of D-galactose, D-mannose, 4-0-β-D-mannopyranosyl-D-mannose, 6-0-α-D-galactopyranosyl-D-mannose, 6-0-α-D-galactopyranosyl-D-mannose and unhydrolysed trisaccharide.

The trisaccharide (50 mg) was dissolved in water 20 ml, cooled to 0°C and added cold solution of sodium metaperiodate (0.2 M, 10 ml). After making up the volume to 50 ml the flask was stored in a refrigerator at 5°C. Periodate consumption and formic acid liberation were constant after 45 hours corresponding to 7.1 moles and 4.0 moles per mole of the trisaccharide, respectively. Table shows the periodate consumption and formic acid liberation at various intervals of time (Fig.31)
Table
Periodate Oxidation of the Trisaccharide A

<table>
<thead>
<tr>
<th>Time (Hours)</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periodate Consumption (in moles per mole of trisaccharide)</td>
<td>4.8</td>
<td>5.9</td>
<td>6.4</td>
<td>6.9</td>
<td>7.1</td>
<td>7.1</td>
</tr>
<tr>
<td>Formic Acid Liberation (in moles per mole of trisaccharide)</td>
<td>2.2</td>
<td>3.1</td>
<td>3.7</td>
<td>3.9</td>
<td>4.0</td>
<td>4.0</td>
</tr>
</tbody>
</table>

Fraction 4
Trisaccharide 'B', 4-0-(4-0-B-D-mannopyranosyl-B-D-mannopyranosyl) - B - D - mannose

The trisaccharide B was obtained as a syrup, yield 60 mg. The syrup had $\left[\alpha\right]_{D}^{25} -15^\circ C$ (in water), (lit. $-16^\circ$, $-22^\circ$ [36]). The syrup had Rgal: 0.34 in solvent (F). The trisaccharide 10 mg was hydrolysed with N-sulphuric acid (2 ml) for 10 hours at boiling water bath. The syrup obtained after usual treatments, subjected to paper chromatographic analysis using solvent (I), showed the presence of D-mannose, 4-0-β-D-mannopyranosyl-D-mannose and unhydrolysed trisaccharide.

Fraction 5
Tetrasaccharide

It was obtained in traces and found to be a mixture of tetrasaccharide as evident rate of movement of the sugar on paper. It could not be analysed further due to paucity of the material.