PART III

SECTION (A) GENERAL METHODS AND TECHNIQUES

SECTION (B) EXPERIMENTAL

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GENERAL METHODS AND TECHNIQUES

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1. Evaporation :

Unless otherwise stated all evaporations were carried out under reduced pressure at low temperature (40-50^{o}C).

2. Neutralisation :

Unless otherwise stated neutralisation of the aqueous acidic solutions were carried out by slow addition of a slurry of barium carbonate under stirring.

3. Optical Rotation :

Optical rotations were determined in a standard polarimeter using sodium lamp as light source. All the values reported are equilibrium values.

4. Sulphated Ash Determination :

A known quantity (0.225 g) of the sample was taken in a weighed silica crucible, moistened with a few drops of concentrated sulphuric acid (A.R. grade) and ignited gently, until well carbonised, on a nonluminous flame. The ignited mass was again moistened with acid as above and heated strongly when the fumes ceased to come out. It was further placed in a muffle furnace and ignited at 600-650^{o}C for 2-3 hours until a constant weight was obtained.

5. De-ionisation :

Deionisation was carried out with freshly regenerated cation exchange resin [Amberlite IR-120 (H^+)] and anion exchange resin [Dowex -3 (OH^-)].

For the purpose of regeneration, the water soaked resins, were poured in pyrex glass tubes (75 x 2.5 cms) fitted with a glass wool at the bottom until a column height of 20 cms was obtained. The columns were washed with hydrochloric acid (2N, 150 ml) in case of cation exchange resins and with sodium hydroxide (2N, 150 ml) in the case of anion exchange resin. During washing, the rate of flow of effluent was maintained at 2 drops/sec. The resin columns were then washed with water repeatedly until the effluent became neutral (pH, 6.5-7.0).

6. Melting Point :

All melting points are uncorrected and determined by Gallen Kamp melting point apparatus.

7. Infrared Spectral Analysis :

The infrared spectral measurements were carried out with a Perkin-Elmer Infrared spectrophotometer Model 137-B. The samples were analysed in any of the following forms :

(a) as pellets of potassium bromide

(b) as solution in dry chloroform.

8. Nuclear Magnetic Resonance Spectral Analysis :

N.m.r. experiments were conducted in the Fourier-transform mode

at 25 MHz for ^{13}C, and at 100 MHz for ^{1}H, with a JEOL FX 100 spectrometer; spectra were recorded for solutions of the polysaccharide in D_2O (32 mg/0.4 ml for ^{13}C- and 10 mg/0.4 ml for ^{1}H-N.m.r.) at 90°. Chemical shifts are expressed relative to internal sodium 4,4-dimethyl-4-silapentanoate -d_4.

9. Paper Electrophoresis :

Electrophoresis of the polysaccharide was conducted on Whatman No.1 MM filter paper sheet in borate buffer (0.05 M sodium tetraborate decahydrate, pH, 9.2) at 400 V and 19 mA for 2.5 hours using Laboratorium Felzercisk model DE-201 apparatus.

10. Chromatographic Techniques :

(a) Paper Chromatography :

Partition chromatography on the filter paper sheets was carried out by desending method[1,2]. For separation of small quantities of sugar mixture, Whatman No. 1 MM chromatographic papers were used and solvent was allowed to flow along the machine line direction as indicated on the paper sheets. For separation of the large quantity (upto 150 mg), Whatman No.3 MM filter papers were used. The following solvent system (V/V) was employed for partition chromatography.

S_1 - n-Butanol-ethanol-water (4:1:5, upper layer)[3]

The following spray reagents were used for detecting sugars :

R_1 - Sodium metaperiodate-Benzidine B[4]

R_2 - Acetonical Silver Nitrate-Alcoholic Sodium hydroxide[5]

R_3 - p-Anisidine phosphate[6]

In case of reagent R_2, the colour spots appeared after heating the paper at 100°C for 2-3 minutes. R_{TMG} values refer to the rates of movement of sugars on paper-chromatogram with respect to 2,3,4,6-tetra -O-methyl-D-glucose.

(b) Column Chromatography :

This was carried out by using a column of cellulose (Zellulose pulver Nr. 123; Carl Schlucher and Schull)[7,8].

Eluant used for cellulose column was n-Butanol half saturated with water (E_1)[7].

(c) Gas-Liquid Chromatography :

The g.l.c. analyses of aldilol acetates of neutral sugars and methylated sugars were conducted using the following conditions.

C_1 - OV-351; 209°C; Flow rate of Nitrogen, 30 ml/min.

C_2 - 3% OV-225; 200°C; Flow rate of Nitrogen, 30 ml/min.

11. Dialysis :

The solutions were dialysed through a cellophane paper against running distilled water at room temperature.

12. Pentosan Estimation[9] :

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

A known amount of the polysaccharide (0.25 g) was accurately weighed and taken in a round bottom flask with a distillation head and a dropping funnel. Hydrochloric acid (100 ml, 12%) was added to the substance and the flask was heated in waxbath at 160-70°C. The distillate was collected at such a rate that when 30 ml of the distillate was obtained in 10 minutes, 12% hydrochloric acid (30 ml portions) was added after every 10 minutes from the dropping funnel. The process was continued until a drop of distillate gave no pink colouration with aniline acetate paper (prepared by mixing equal volumes of aniline and water, adding glacial acetic acid dropwise and shaking until the milkiness disappeared). The distillate was treated with an excess of phloroglucinol solution* when a greenish-black precipitate appeared. The volume of the solution was made upto 400 ml with hydrochloric acid (12%) and left for overnight. The precipitated furfural phloroglucide was filtered through a weighed sintered glass crucible and washed with 150 ml water, care being taken to see that the residue does not dry up till the very end. It was finally washed with 95% ethanol and dried in an oven at 105°C to a constant weight. From the weight of the phloroglucide, furfural, pentoses and pentosans contents were calculated from the Krober's Table[10].

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* The phloroglucinol solution was prepared by heating hydrochloric acid (100 ml, 12%) with phloroglucinol (3.6 g). The hot solution was made upto 500 ml with hydrochloric acid (12%) and allowed to stand overnight. The solution was filtered immediately before use.

13. Determination of Methoxyl Content :

In this method, the sample is heated with boiling hydriodic acid whilst a slow stream of an inert carrier gas is passed through the reaction mixture. The methyl iodide formed from the methoxyl groups in the sample is carried by the stream of gas into an absorption vessel containing a solution of sodium acetate and bromine in glacial acetic acid, where it decomposed and the iodide oxidized to iodate by the bromine. After the addition of formic acid to remove excess of bromine from the solution, sulphuric acid and potassium iodide are added, and the liberated iodine is titrated with standard sodium thiosulphate using starch as indicator. The apparatus used is a modified Clark's apparatus[11] for volumetric procedure which consists of a reaction flask with side arm, condenser with scrubber inlet tube and volumetric receiver.

$$ROCH_3 + HI = CH_3I + ROH$$

$$CH_3I + 3\ Br_2 + 3\ H_2O = CH_3Br + HIO_3 + 5\ HBr$$

$$Br_2 + HCOOH = 2\ HBr + CO_2$$

$$IO_3^- + 5\ I^- + 6\ H^+ = 3\ I_2 + 3\ H_2O$$

$$3\ I_2 + 6\ (S_2O_3)^{--} = 3\ (S_4O_6)^{--} + 6\ I^-$$

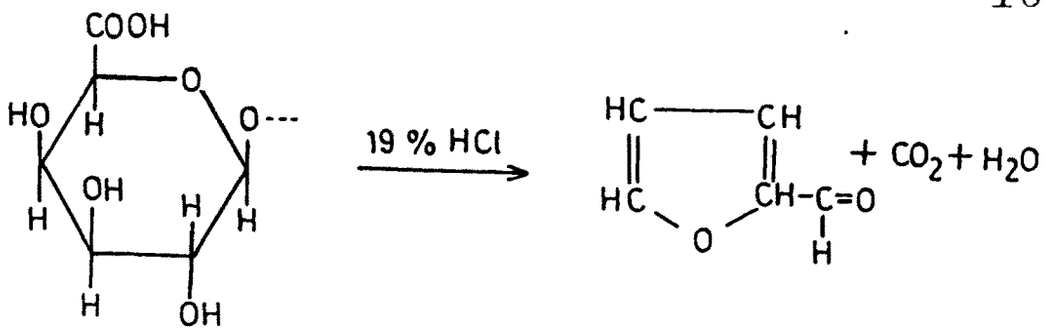
The substance (8.0 mg) was accurately weighed in the foil and placed into the reaction flask in which phenol (0.2 ml), acetic anhydride (0.4 ml) and hydroiodic acid (5 ml) were added. The side tube of the reaction

flask was fitted to the Kipp's carbon dioxide generator and the outlet from the condensor was allowed to dip into the receiver containing a solution of bromine (0.5 ml) in glacial acetic acid and 10 ml of 20% sodium acetate solution. The reaction flask heated in a wax bath at 130-135^{o}C and carbon dioxide gas was allowed to pass at a slow rate. At the end of the experiment, the contents of the receiver and washings were transferred to a stoppered Erlenmeyer flask containing aqueous sodium acetate (20%, 10 ml). Formic acid was added drop by drop till the colour of bromine disappeared and the vapour of excess bromine was removed by aeration. Potassium iodide (1 g) and 2 N sulphuric acid (5 ml) were added. The flask was stoppered, shaken and allowed to stand for five minutes in dark. The liberated iodine was then titrated against standard sodiumthiosulphate (0.025 N). The percentage of methoxyl content was calculated by means of the following equation.

$$\% \text{ Methoxyl} = \frac{\text{ml of 0.025 N sodium thiosulphate solution} \times 0.129 \times 100}{\text{Wt of the substance (mg)}}$$

14. Determination of Uronic Acid[12] :

The method is based upon the fact that the uronic acids, upon oxidation with hydrochloric acid, undergo decarboxylation giving rise to a quantitative yield of carbon dioxide as given below :



The substance (0.2 g) was taken in the reaction flask and hydrochloric acid (19%, 30 ml; A.R. grade) was added to it with a few porcelein pieces to prevent bumping at the start of analysis, dry nitrogen gas was passed rapidly for 15 minutes to remove all CO_2 gas from the apparatus and trap. Sodium hydroxide (0.25 N, 50 ml) and 5 drops of 1-butanol were added to the lower of the previously swept absorption system and this assembly including short sodalime tube was connected to the first part of the apparatus. Reaction flask was heated in a oilbath to 145^{o}C for 2 hours. In order to minimise undesirable decomposition of the polysaccharide, the reaction flask was allowed to dip in the oilbath upto the level of hydrochloric acid. After 2 hours, heating was discontinued but the nitrogen supply was maintained as before. The absorption assembly was disconnected & contents of the tube was quantitatively transferred into a flask using CO_2 free water. Barium chloride solution (10%, 10 ml) was added to the flask and the excess of alkali was titrated with 0.1 N hydrochloric acid to the phenolphthalein as indicator.

Normal precautions were taken to avoid the contamination of atmospheric carbon dioxide. The volume of acid required was substracted from the corresponding volume required in complete blank run. The

difference (net volume) of acid provides a measurement of anhydrouronic acid content according to the following equation.

$$\text{A.U.A. (\% W/W)} = \frac{8.8 \times \text{Normality of HCl} \times \text{Net volume of acid (ml)}}{\text{Weight of the sample (g)}}$$

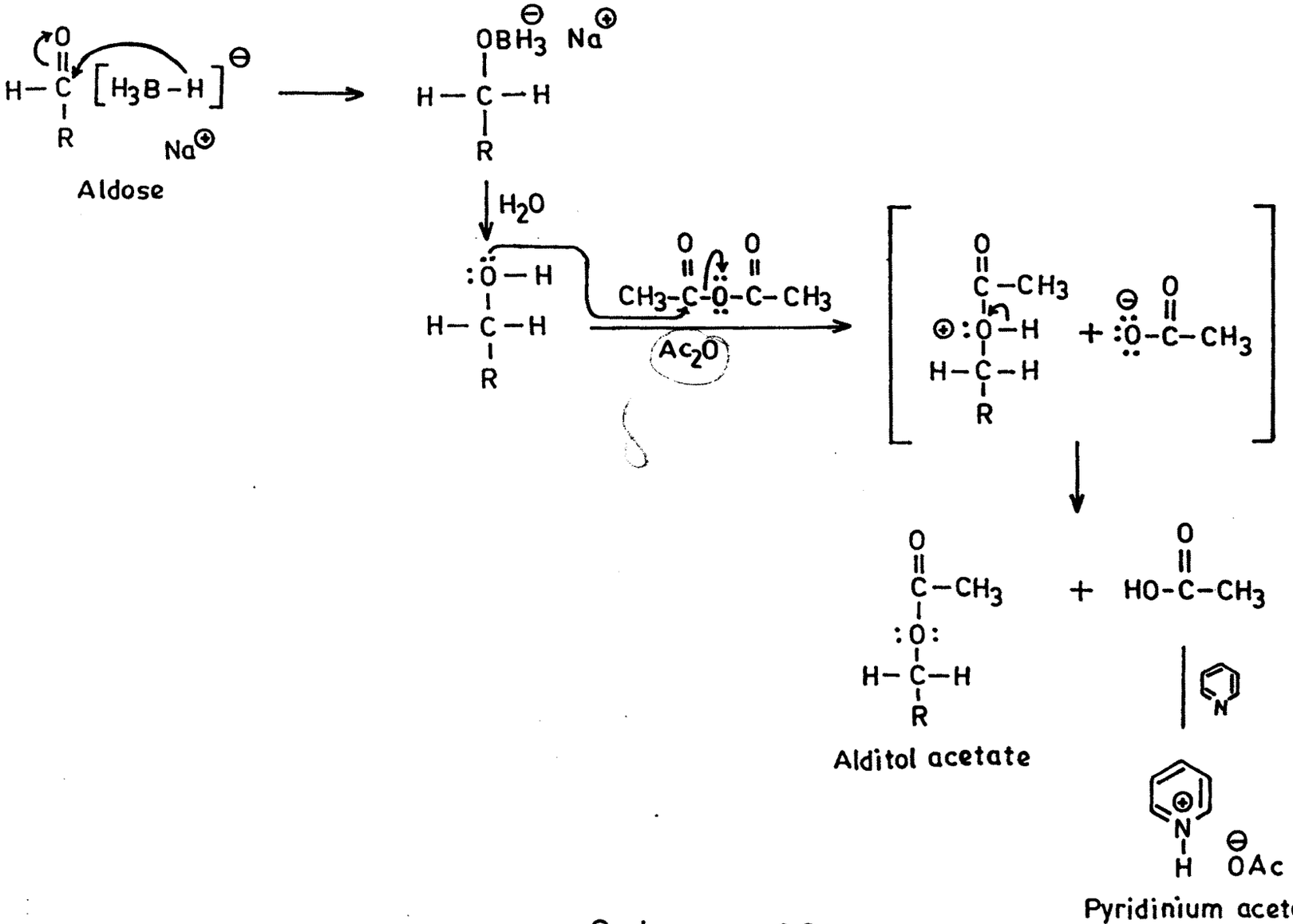
15. Hydrolysis :

Hydrolysis of the material was carried out with dilute acid under the following conditions depending upon the extent to which the degradation of the polysaccharide was to be carried out.

Complete Hydrolysis : It was effected by heating the polysaccharide with dilute sulphuric acid (2N) at 100^{o} to achieve its complete depolymerisation. The course of the hydrolysis was followed by iodometric titration.

16. Preparation of Alditol Acetate Derivatives[13] :

The known amount of hydrolysate was reduced with sodium borohydride. After neutralisation with Amberlite IR-120 (H^{+}), the filtrate was evaporated and boric acid was removed by the repeated addition and evaporation of methanol. Then the products were acetylated with acetic anhydride-pyridine mixture (1:1) at $100^{o}C$ for 30 min. After evaporation of the solution, the residue was dissolved in chloroform-methanol mixture (1:1) for g.l.c. analysis. The mechanism of the reduction and acetylation of the sugar is depicted below (Scheme 1.3).



Scheme – 1·3

FORMATION OF SUGAR ALDITOL ACETATE DERIVATIVE

17. Mass Spectral Analysis :

Mass spectral analysis of alditol acetates of methylated sugars was carried out using the following GLC-MS model.

JEOL model JGC-20K gas chromatograph and JMS-D100 mass spectrometer.

The characteristic m/z values of different fragments were noted.

18. Determination of Sugar Ratio in the Polysaccharide :

For quantitative separation of sugar mixture into its individual components, obtained as a result of acid hydrolysis, a large filter paper sheet of Whatman No.1 or No. 3 MM was taken and a starting line was drawn at a distance of 4" along the direction of machine line. The paper was divided into five different parts (vertically) in such a manner that the two strips at the edges and the central one were of equal width ($1\frac{1}{2}$"). The small strips were used as a guide and the spot of sugar mixture containing a known amount of D-ribose were placed at their centres, while on the other two big strips, the spots were placed 0.5 cm apart from the starting line by means of fine capillary. After drying the spotted end, the paper was immersed in the desired solvent in a trough placed inside a chromatographic chamber. After allowing the chromatogram to run for several hours, it was taken out and dried in air. The three small strips were cut and sprayed with reagent R_1 in the usual manner to locate the spots of individual sugar moieties.

The chromatogram was then reassembled and the areas on the unsprayed central portions of the chromatograms, containing the individual

components were cut. The individual paper strips were eluted with water in a closed chamber according to Dent's procedure[14]. One end of the paper strip to be eluted was placed between the two glass slides. The other end of the slides were put into petri dishes containing water and the drops of water coming out from the lower end of the vertically hanging paper strips were collected. In this way the individual sugars were collected and afterwards estimated by periodate oxidation method and also by phenol-sulphuric acid method as follows.

(a) Periodate Oxidation Method[15] :

The aqueous solution of the individual sugar was taken in a stoppered flask, mixed with freshly prepared sodium metaperiodate (0.25 M, 1 ml) and heated on a boiling water bath for 20 minutes. The flask was cooled and ethylene glycol (0.2 ml) was added to destroy excess of sodium meta-periodate. The liberated formic acid was titrated iodometrically[16,17].

(b) Phenol-Sulphuric Acid Method[18,19] :

This method depends upon the reaction of the sugar moieties with concentrated sulphuric acid in presence of aqueous phenol. The reaction is exothermic and thereby increases the temperature of the contents. A quantitative estimation of the individual sugar can be carried out colori-metrically since a yellow-orange coloured solution is produced.

The aqueous solution (9-60 ug) of the individual sugar was taken in test tubes and the volume of each was adjusted to 2 ml by addition of distilled water. It was mixed with 1 ml of aqueous phenol solution (5% W/V). After keeping the test tubes for 10 minutes, sulphuric acid

(A.R., 5 ml) was then added rapidly in a stream, taking care that the acid should not touch the walls of the test tubes. The resulting solution after thorough shaking was allowed to stand for 10 minutes at room temperature and then cooled by placing in cold water. The absorbance of the yellow-orange colour was measured at 490 n.m. in spekol against a water blank containing same amount of reagents. From the absorbance value, the sugar content was determined by referring to a standard curve prepared with a pure sample of D-galactose and D-mannose.

Procedure for Drawing Calibration Curve for D-Galactose and D-mannose :

The standard curve for individual sugars, D-galactose and D-mannose were drawn by plotting the amount of sugars against absorbance values. Sugar sample (45 to 50 mg) was taken in one litre volumetric flask and the volume was made upto the mark with distilled water. In order to estimate the sugar, aliquots containing (9-60 ug) of the sugar was pipetted out from each flask into a series of six test tubes and the volume of each adjusted to 2 ml by addition of calculated amount of water. It was then mixed with 1 ml of aqueous phenol solution (5% W/V). After 10 minutes, sulphuric acid (A.R., 5 ml) was added rapidly, the stream of acid being directed against the liquid surface rather than against the side of the test tubes in order to obtain a good mixing. The tubes were allowed to stand for 10 minutes at room temperature and shaken well and cooled in running water. A blank experiment was also performed in a similar way. The absorbance of yellow-orange colour developed in test tubes containing sugar solution was measured at 490 n.m. in spekol. The analytical results (Table

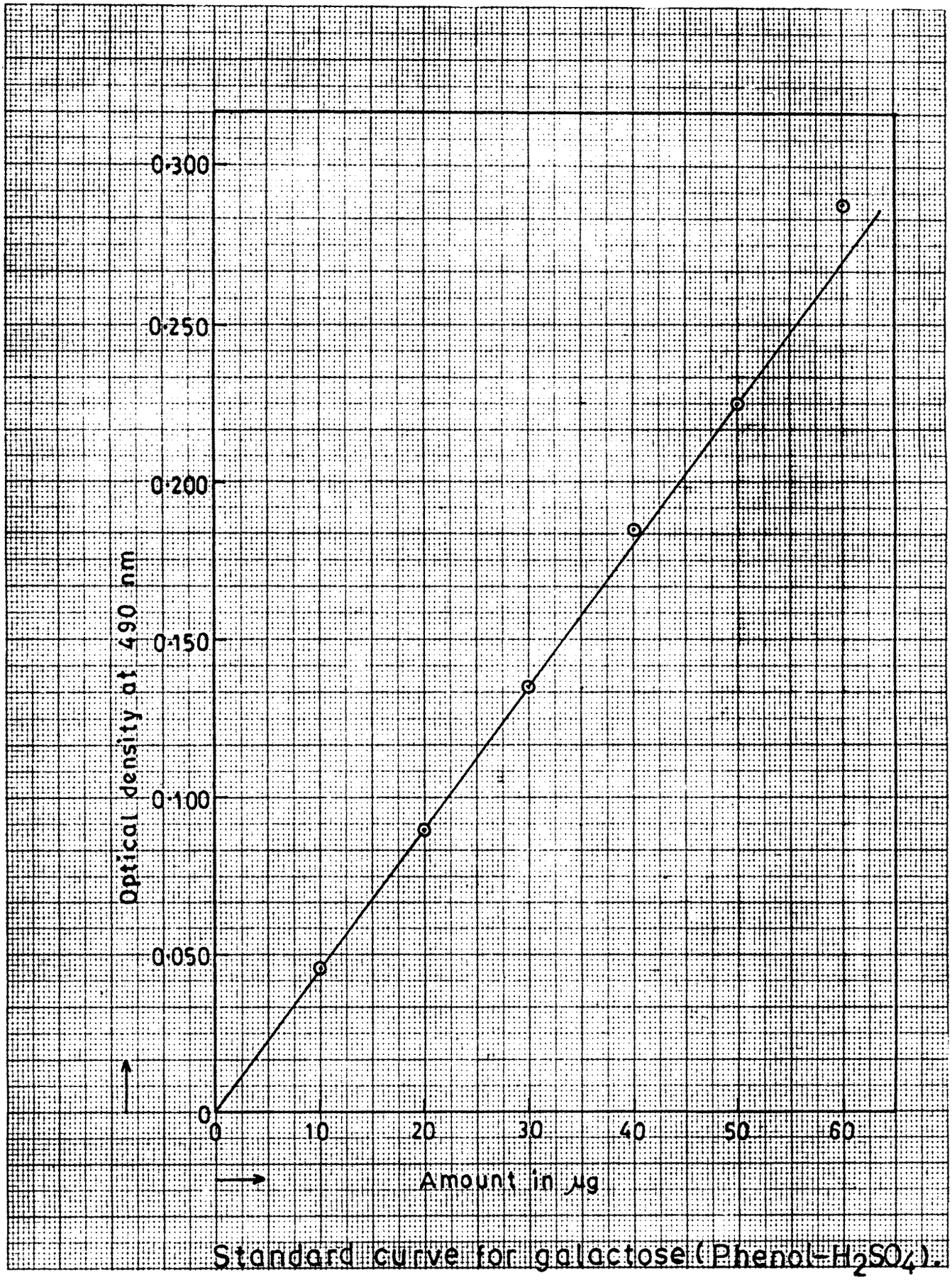
1.8 and 1.9) were then plotted to get the standard curve (Figs. 1.12 and 1.13) for galactose and mannose respectively.

Table 1.8

Absorbance Values for D-Galactose at Different Concentrations

(0.05 g D-galactose dissolved in 1 litre water)

Sl. No.	Galactose (in ug)	Phenol (5%,W/V) (in ml)	Conc. Sulphuric acid (in ml)	Optical density (O.D.) at 490 nm
1	0	1	5	0.170 (adjusted to zero)
2	10	1	5	0.045
3	20	1	5	0.090
4	30	1	5	0.135
5	40	1	5	0.185
6	50	1	5	0.225
7	60	1	5	0.288



Standard curve for galactose (Phenol-H_2SO_4).

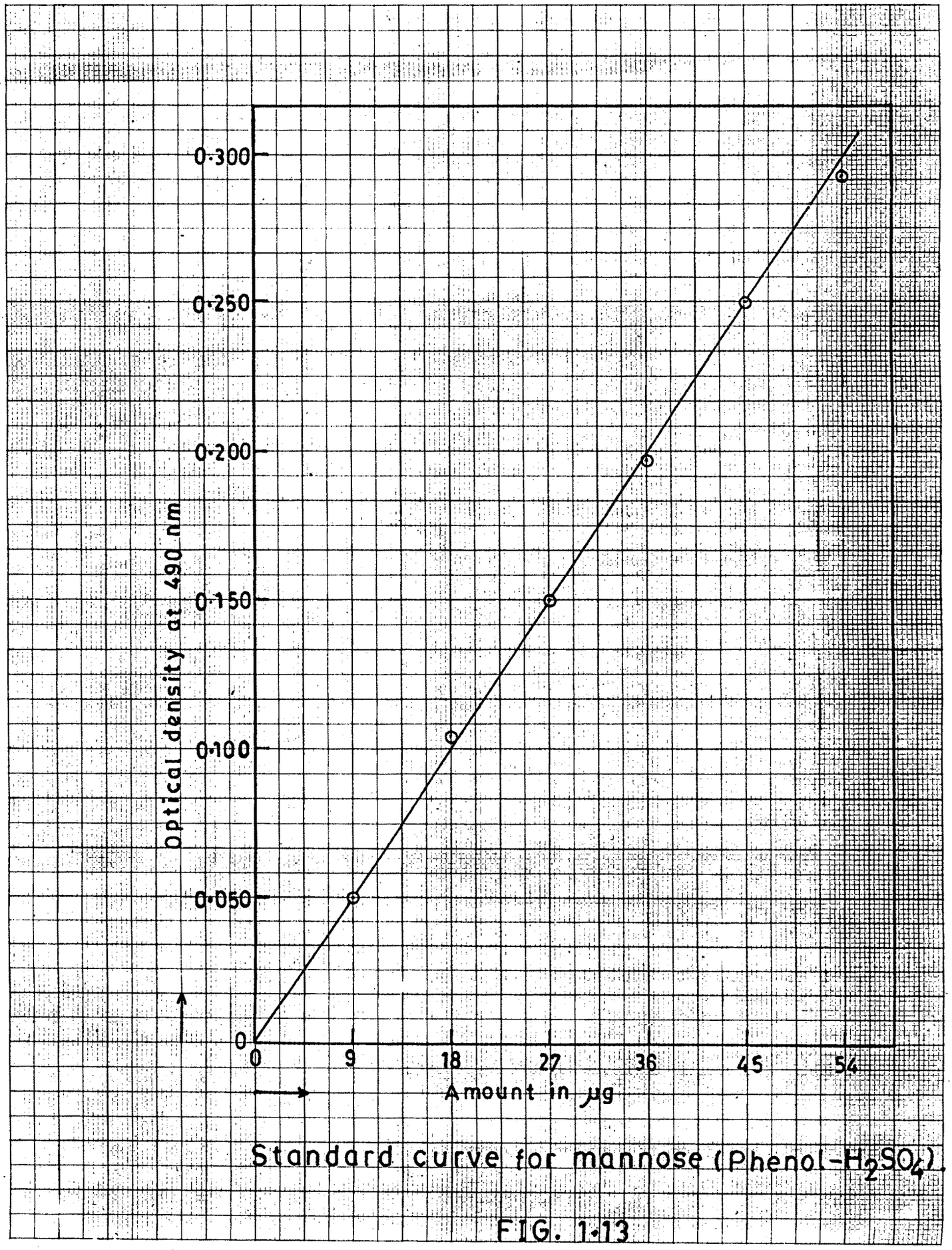
FIG. 1·12

Table 1.9

Absorbance Values for D-Mannose at Different Concentrations

(0.045 g D-mannose dissolved in 1 litre water)

Sl. No.	Mannose (in ug)	Phenol (5%,W/V) (in ml)	Conc. Sulphuric acid (in ml)	Optical density (O.D.) at 490 nm
1	0	1	5	0.170 (adjusted to zero)
2	9	1	5	0.050
3	18	1	5	0.105
4	27	1	5	0.150
5	36	1	5	0.198
6	45	1	5	0.250
7	54	1	5	0.293



Standard curve for mannose (Phenol-H_2SO_4).

FIG. 1·13

(c) Gas Chromatographic Method :

This technique is most effective in the quantitative analysis of the mixture of volatile compounds. The number of peaks recorded reflect the number of components in the sample. Furthermore, as a first approximation it can be assumed that the peak area of a given compound in its vapour phase chromatography will be directly proportional to its weight percentage in the original mixture. Peak area may be measured in a number of ways as indicated below :

(i) By an electronic integrator connected to the recorder.

(ii) By cutting out the peaks and weighing the pieces of the paper on an analytical grade balance. (This is possible because the recorder paper has a very uniform thickness and density throughout its length and breadth).

(iii) By a planimeter which can be used to measure the area under each peak.

(iv) The peak area can be calculated by the following relationship.

Area = peak height x width at half height

when the peaks are symmetrical and well separated from one another.

In the present investigation, GLC technique was also applied to perform quantitative analysis of the sugars by calculating the ratio of sugars relative to their peak areas. Peak area of individual sugar was measured either by an electronic integrater connected to the recorder or by relationship as described in (iv) as above.

19. Demethylation of O-Methylated Sugars[20] :

The methylated sugar (5-10 mg) was taken in a test tube and 48% hydrobromic acid (1-2 ml) was added. The tube was sealed and heated in a boiling water bath for five minutes. It was taken out, cooled and the seal was broken. The contents were diluted with water, neutralised with freshly prepared silver carbonate and left for 4 hours. The precipitate of silver bromide and excess of silver carbonate were removed by filtration and washed with water. Hydrogen sulphide was passed into the combined filtrate and the precipitate of silver sulphide was removed by filtration and washings were evaporated to obtain demethylated sugar in the form of thin syrup.

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PART III

SECTION (B)

EXPERIMENTAL

Preliminary Analysis of the Seeds of Adenanthera pavonina

(1) Moisture Content of the Seeds :

Powdered seed (0.26 g) was taken in a weighed crucible and was placed in a vacuum oven at 60-70^{o}C for 6 hours until a constant weight was obtained. The moisture content of the seeds was found to be 8.9%.

(2) Ash Content of the Seeds :

Powdered and dried seed (0.72 g) was analysed for its sulphated ash content according to the procedure described earlier. The ash content of the seeds was observed to be 3.72%.

(3) Estimation of Proteinous Matter :

The powder of the seeds (0.4280 g) was transferred to a clean and dry Kjeldahl flask. To this was added about 1 g catalyst mixture [1:20 mixture of cupric sulphate (A.R. grade) and potassium sulphate (A.R. grade)] and 30 ml conc. sulphuric acid (A.R. grade). The flask was fitted with loose glass-stopper and heated for 5 hours till the original colour of the reaction mixture disappeared. The Kjeldahl flask was then cooled and its content diluted with distilled water (40 ml) and then carefully transferred to the round bottom flask of the distillation unit. Finally, the Kjeldahl flask was washed with water several times and the washings transferred to the round bottom flask. The flask was fitted with the dropping funnel and Kjeldahl trap attached to the condenser with its lower end dipping in sulphuric acid solution (0.1 N, 50 ml). Then 40% sodium hydroxide solution (50 ml) was introduced from the dropping funnel taking

care to leave some solution in the funnel which serves as liquid seal. The flask was heated directly. The ammonia evolved was absorbed in sulphuric acid solution (0.1 N, 50 ml) taken in a 250 ml flask. After completing the reaction, the receiver was removed and the excess of acid was back titrated with standard sodium hydroxide solution (0.1 N). The percentage of nitrogen and protein of the seeds were found to be as follows :

(i) Percentage of nitrogen = 3.72

(ii) Percentage of proteinous matter = 3.72 x 6
= 22.32.

(4) Oil Content of the Seeds :

Powdered and dried seeds (100 g) was Soxhlet extracted with petroleum ether (80-100^{o}C) for 15 hours on boiling water bath. The petroleum ether extract after evaporation furnished an oily mass (yield 12.9%).

Isolation and Purification of the Polysaccharide Occurring in the Seeds of Adenanthera pavonina :

The matured, Adenanthera pavonina seeds were cleaved in a low speed grinder when the endosperm together with seed coat broken-off from the cotyledons. The broken material after dehusking was Soxhlet extracted with petroleum ether (80-100^{o}C) for 15 hours on boiling water bath to remove its oil content. The defatted material (100 g) was soaked in water (1.5 l) and left overnight. The swollen material was then heated at 50^{o}C for 12 hours under constant stirring and mixed with additional quantity of water. It was filtered through a muslin cloth. The filtrate was centrifuged at 3000 r.p.m. for 30 minutes and the supernatant solution was decanted. This, upon acidification with glacial acetic acid produced turbidity. This

was centrifuged again at 3000 r.p.m. for 30 minutes and finally filtered through Kiesulghur bed to furnish a clear solution. This solution was concentrated (1 l) and poured in a thin stream into large excess of ethanol (4 l) with constant stirring to furnish crude polysaccharide in the form of creamy white precipitate (Chart-1.2). After decantation of aqueous ethanol, the precipitate was treated with acetone and finally with absolute alcohol until the creamy white precipitate became granular in nature (yield, 12.4 percent).

The crude polysaccharide was purified by

(1) Cation exchange treatment and Dialysis, followed by fractionation via

(2) Copper complex formation.

(1) Cation Exchange Treatment and Dialysis :

In this process, the aqueous solution of the compound (12 g in 850 ml water) was passed through the column of freshly regenerated cation exchange resin [Amberlite IR-120 (H^+)]. The column was washed with water until the effluent showed a negative test for carbohydrate. The effluent was dialysed under running distilled water for 72 hours and concentrated to 600 ml. The resulting solution (600 ml) was precipitated with ethanol (95%, 4 volumes). The precipitate was filtered & washed with absolute alcohol, and dried by solvent exchange as described above, followed by placing the polysaccharide material in desiccator under vacuum to give purified polysaccharide (Chart-1.3).

(2) Copper Complex Formation :

In this process, Fehling's solution was slowly added to the aqueous solution of the polysaccharide (7.92 g in 400 ml). As the addition continued, the viscosity of the solution increased considerably and then it decreased as soon as the copper complex was precipitated. The precipitate was separated by centrifugation and the supernatant solution was again treated with Fehling's solution until there was no precipitate. The polysaccharide was regenerated from the greenish blue copper complex by suspending it in ice-cold water (270 ml) and treating with N-hydrochloric acid under constant stirring until the decomposition was complete. Acidified ethanol (4 volumes) was then added to the solution slowly and the precipitate was collected, washed with absolute alcohol and dried under reduced pressure.

The dried polysaccharide (as obtained from Cu complex formation process) was dissolved in the minimum quantity of water, deionised by passing through the columns of freshly regenerated cation exchange resin [Amberlite IR-120 (H^+)] and anion exchange resin [Dowex-3 (OH^-)] successively. The purified and fractionated polysaccharide was precipitated from the final effluent with four volumes of ethanol, filtered to collect the precipitate and dewatered by solvent exchange with acetone, followed by absolute ethanol, and finally dried in a vacuum desiccator at room temperature to furnish white fibrous powder of the purified and fractionated polysaccharide (yield, 8.2 percent) (Chart-1.3).

The purified and fractionated polysaccharide dissolves slowly in water to form clear solution, almost neutral in character (pH 6.6). It was free from starch as indicated by the absence of blue colour with iodine

solution and did not reduce Fehling's solution or Tollen's reagent. Nitrogen, sulphur, halogens, methoxyl, pentoses and anhydrouronic acid groups were found to be absent. Its specific rotation showed a value $[\alpha]_D^{30}$ + 71.02° (C, 0.09 in water).

Homogeneity of A. pavonina Seed Polysaccharide :

The analysis of the polysaccharide by electrophoresis was carried out on Laboratorium Felzerdsk model DE-201 apparatus. A 1.0% solution of the polysaccharide was subjected to electrophoresis using Whatman No. 1 MM filter paper sheet, in 0.05 M sodium tetraborate decahydrate buffer (pH, 9.2) at 400 V and 19 mA for 2.5 hours. After electrophoretic run, the paper strip was taken out, dried and sprayed by the staining reagent R_1. On paper electrophoresis, the polysaccharide migrated as a single spot on the paper strip (Fig. 1.3).

Infrared Spectral Analysis :

The infrared spectrum of the purified and fractionated polysaccharide taken in potassium bromide pellets showed a strong band for -OH (3400 cm^{-1}) along with other bands at 2920, 1640, 1155, 1070, 1030, 875 and 817 cm^{-1}.

Nuclear Magnetic Resonance Spectral Analysis :

^{13}C- and ^{1}H-N.m.r. spectra of purified and fractionated polysaccharide in D_2O (32 mg/0.4 ml) and (10 mg/0.4 ml), were recorded at 25 and 100 MHz respectively. In ^{13}C-N.m.r. spectrum, the low field values of δ 101.8 and 103.0 were observed for the C-1 signals of α-D-galactopyranosyl and ß-D-mannopyranosyl units, respectively. The ^{1}H-N.m.r.

spectrum of the polysaccharide contained a signal at δ 4.8 ($J_{1,2} \sim$ 1.0 Hz) from the anomeric proton of ß-D-mannopyranosyl units, and a doublet δ 5.0 ($J_{1,2} \sim$ 3.0 Hz) from the anomeric proton of α-D-galactopyranosyl units. This is compatible with the expected $^4C_1$ conformation of α-D-galactopyranose and ß-D-manopyranose rings.

Sulphated Ash Content of the Polysaccharide :

The samples of crude (0.1556 g) and pure (0.2204 g) A. pavonina seed polysaccharide were analysed for their sulphated ash content. The results are given below :

Sulphated ash in the crude polysaccharide = 7.80%

Sulphated ash in the pure polysaccharide = 0.12%

Complete Acid Hydrolysis of A. pavonina Seed Polysaccharide and Characterisation of the Cleavage Products :

Complete acid hydrolysis of pure polysaccharide was performed by using dilute sulphuric acid.

(1) Complete Acid Hydrolysis of the Polysaccharide with 2 N Sulphuric Acid

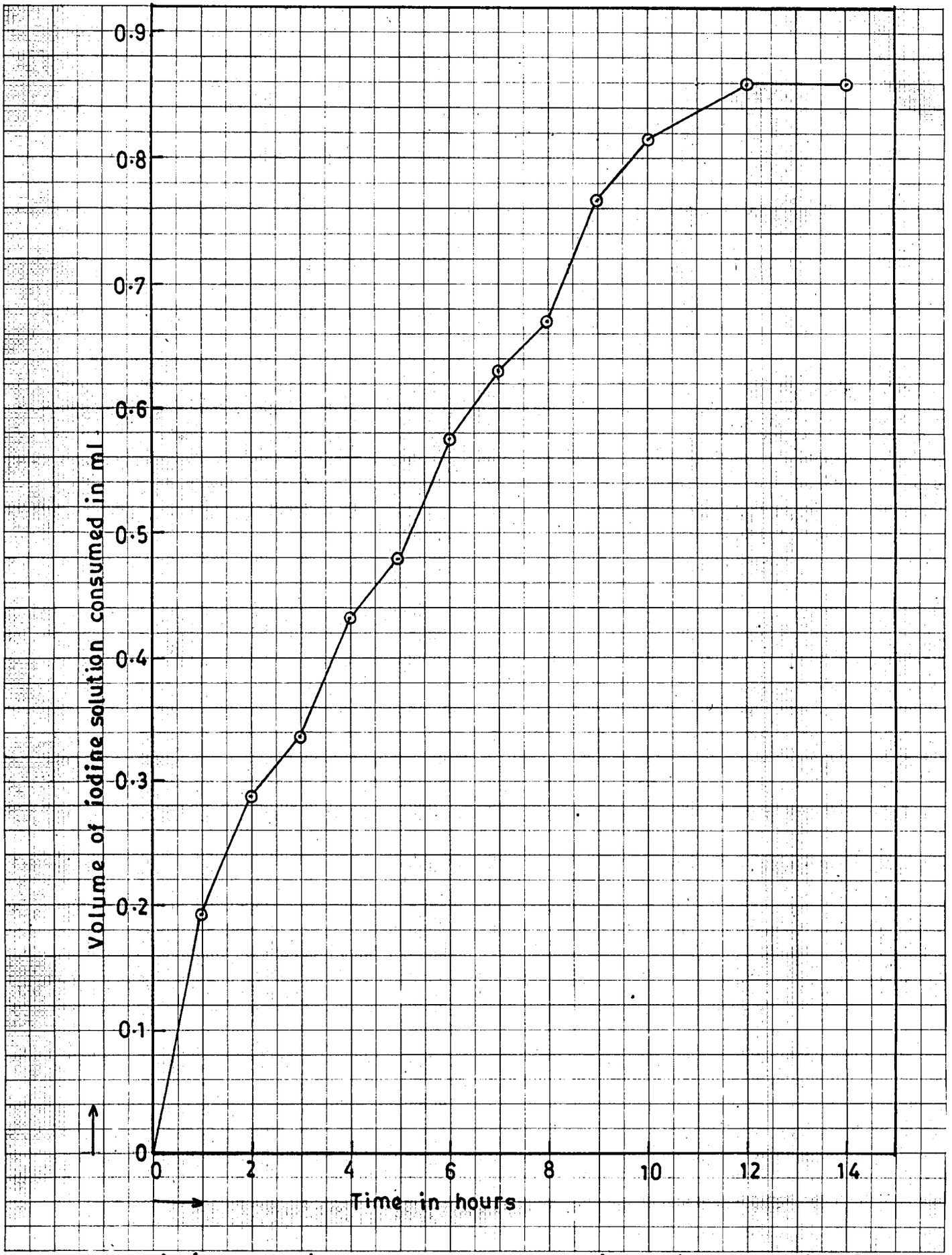
Purified and fractionated polysaccharide (4 g) was subjected to hydrolysis with sulphuric acid (2N, 300 ml) for 12 hours on a boiling water bath. The course of hydrolysis was followed by iodometric titration[21]. After definite intervals of time, an aliquot (2 ml) of the hydrolysate was withdrawn in a Erlenmeyer flask and mixed with iodine (0.1, 20 ml) and sodium hydroxide solution (0.1 N, 30 ml). The mixture was kept for 20 minutes in dark. The solution was then acidified with sulphuric acid (2N, 10 ml) and

excess of iodine was titrated with standard sodium thiosulphate solution (0.05 N). The time taken for completion of hydrolysis along with the variation of iodine absorptions during the progress of the reaction is recorded in Table 1.10 and Fig. 1.14.

Table 1.10

Progress of Acid Hydrolysis of the Polysaccharide with 2 N Sulphuric Acid

Time in hours	Volume (in ml) of iodine mixed with 2 ml of hydrolysate	Volume (in ml) of hypo solution (0.05N) equivalent to excess iodine	Volume (in ml) of iodine solution consumed
0	20	35.85	0.00
1	20	35.65	0.19
2	20	35.55	0.29
3	20	35.50	0.34
4	20	35.40	0.43
5	20	35.35	0.48
6	20	35.25	0.58
7	20	35.20	0.62
8	20	35.15	0.67
9	20	35.05	0.77
10	20	35.00	0.82
12	20	34.95	0.86
14	20	34.95	0.86



Major Acid hydrolysis of polysaccharide with 2N H_2SO_4.

FIG. 1·14

The acid hydrolysate was filtered, neutralised and concentrated to a syrup. It was refluxed with dry methanol (175 ml) in order to ensure the removal of uronic acid as its corresponding barium salts. The clear methanolic extract was concentrated yield a mixture of neutral sugars in the form of syrup (yield, 3.56 g). Preliminary chromatographic examination of the sugar mixture, using solvent S_1 and spray reagent R_2 revealed the presence of two spots having R_f values of 0.07 and 0.11, corresponding to those of D-galactose and D-mannose respectively. These sugars also shows their movements identical to those of standard sugars on paper chromatogram using same solvent system (S_1).

Resolution of Neutral Sugar Mixture into its Components by Cellulose Column Chromatography :

Further resolution of the neutral sugar mixture (2.68 g approx.) into its individual components was carried out by subjecting it to chromatographic separation on a column of cellulose. Elution of the column was made with solvent E_1 and several fractions (10 ml each) were collected, using an automatic fraction collector. Each fraction was subsequently examined paper chromatographically for the detection of sugar (solvent S_1, spray reagent R_2). The appropriate fractions of the individual sugar were then combined and evaporated to dryness to obtain pure specimen of sugar moieties. The results of chromatographic analysis are summarised in Table 1.11.

Table 1.11

Resolution of Neutral Sugar Mixture by Cellulose Column Chromatography

Fraction No.	R_f (in solvent S_1)	Yield (in g)	Remarks
1-52	-	-	No sugar
53-95	0.11	0.63 g	Mannose
96-132	0.11 and 0.07	1.14 g	Mannose and Galactose
133-178	0.07	0.59 g	Galactose

Characterisation of the Sugar Fractions from the Hydrolysate of the Polysaccharide by Classical Methods :

Sugar Fractions (53-95) :

The syrup obtained from the combined fractions (53-95) was crystallised from ethanol (yield, 0.63 g; R_f 0.11 in solvent S_1). It had $[\alpha]_D^{24}$+16.1° (C, 0.54 in water) and m.p. and m.m.p. (with an authentic sample of D-mannose) 131-32°C. The migration rate and $[\alpha]_D$ values of the sugar were found to be in close agreement with respective values[22] reported for D-mannose. The identity of the sugar was further established by preparing its p-nitro-aniline derivative. Sugar (0.21 g) was refluxed with equimolar quantities of p-nitro-aniline in absolute ethanol containing a trace of hydrochloric acid on a steam bath for 20 minutes. The resulting solution was concentrated when crystals of p-nitro-N-phenyl-D-mannosylamine separated out. Upon crystallisation from ethanol, it showed m.p. 217-18°C which remains undepressed on admixture with an authentic sample.

Sugar Fractions (133-178) :

The above fractions (133-178), upon concentration, furnished a syrup, which gave rise to a white granular mass when triturated with ethanol (yield, 0.59 g; R_f 0.07 in solvent S_1). It had an optical rotation $[\alpha]_D^{24}$ $+81.02^o$ (C, 0.59 in water) and m.p. and mixed m.p. (with an authentic sample of D-galactose), 165-66°. All the above physical constants agreed with respective values[23] reported for D-galactose. The sugar (0.19 g) upon refluxing with an alcoholic solution of p-nitroaniline containing a trace of hydrochloric acid, furnished crystalline p-nitro-N-phenyl-D-galactosylamine, m.p. and mixed m.p. (with an authentic sample), 213-14°C.

Quantitative Estimation of the Sugars in A. pavonina Seed Polysaccharide :

A. pavonina seed polysaccharide (0.3852 g) was subjected to quantitative hydrolysis with sulphuric acid (2N, 20 ml) for 12 hours in a sealed tube, on a boiling water bath. After completion of the reaction, the tube was cooled and the seal was broken. The contents were then neutralised with a slurry of Barium Carbonate, filtered and the filtrate was concentrated to syrup. This was exhaustively extracted with methanol and a part of the syrup after evaporation was subjected to preparative partition chromatography on Whatman No. 3 MM filter paper sheets (solvent S_1, spray reagent R_2) for the isolation of individual sugar components as described earlier. The aqueous extracts were taken separately in 100 ml volumetric flask and volumes were made upto the mark by addition of water. The sugars were estimated by the following two methods; in addition to this, the sugars were also estimated by g.l.c.:

(1) Periodate Oxidation Method :

The aqueous solution (20 ml) of the neutral sugars, D-galactose and D-mannose, were oxidised separately with sodium metaperiodate (0.25M, 1 ml) by heating on boiling water bath for 20 minutes and the formic acid liberated was estimated iodometrically as described earlier[16,17]. D-galactose and D-mannose were found to be present in an approximate ratio of 1:1.05.

(2) Phenol-Sulphuric Acid Method[18,19] :

The individual sugars were estimated according to the procedure described earlier. The colour intensity of different sugar samples was read at 490 nm in spekol. From the absorbance values, the sugar content of D-galactose and D-mannose were determined by referring to the standard curve (Figs. 1.8 and 1.9). The results of estimations are recorded below (Table 1.12 and 1.13).

Table 1.12

Estimation of Galactose

Sl. No.	Volume of sugar solution (in ml)	Volume of water added (in ml)	Volume of 5% (W/V) phenol (in ml)	Volume of sulphuric acid (in ml)	Optical density (O.D.) at 490 nm	Amount of sugar (in μg)
1	0.2	1.8	1	5	0.083	
2	0.2	1.8	1	5	0.083	18.50
3	0.2	1.8	1	5	0.083	

Table 1.13

Estimation of Mannose

Sl. No.	Volume of sugar solution (in ml)	Volume of water added (in ml)	Volume of 5% (W/V) phenol (in ml)	Volume of sulphuric acid (in ml)	Optical density (O.D.) at 490 nm	Amount of sugar (in µg)
1	0.2	1.8	1	5	0.108	
2	0.2	1.8	1	5	0.108	19.35
3	0.2	1.8	1	5	0.108	

From the respective amount of different sugars,D-galactose and D-mannose was found to be present in an approximate molar ratio of 1 : 1.05.

(3) Gas-Chromatographic Method :

The remaining part of syrup (containing sugar mixture) was converted into its alditol acetate derivative according to the procedure described earlier[13]. The residue was dissolved in chloroform-methanol mixture (1:1) was subjected to g.l.c. analysis under column condition C_1 as described earlier. The g.l.c. part is given in Fig. 1.8. The results in Table 1.3 revealed that the sample was composed ofD-mannose and D-galactose in the molar ratio of 1.05 : 1.00, respectively. For the sake of comparison, g.l.c. of alditol acetates of known sugars was also carried out under the column condition C_1 to obtain their retention times (Table 1.4).

Methylation of Adenanthera pavonina Seed Polysaccharide :

Polysaccharide was completely methylated according to the procedure

of Hœworth[24] followed by Purdie Method[25]. Purified polysaccharide (8.4 g) was dissolved in sodium hydroxide (40% W/V, 350 ml) and dimethyl sulphate (175 ml) was then added in small quantities with vigorous stirring over a period of 7 hours at 5^{o} in an inert atmosphere of nitrogen. After treating the reaction mixture with four more lots of sodium hydroxide (30% W/V, 120 ml) and dimethyl sulphate (60 ml), the resultant product was heated carefully on a steam bath for 2 hours to decompose the excess of dimethyl sulphate. The mixture was cooled, neutralised with cold sulphuric acid (1:4, V/V) and finally acidified with glacial acetic acid. The precipitated sodium sulphate was filtered and the residue was extracted exhaustively with chloroform. The chloroform extract after evaporation was treated with water and mixed with filtrate. The combined extract was dialysed against running distilled water for 72 hours. The dialysed solution was concentrated (100 ml) and extracted with chloroform in a liquid-liquid extractor for 16 hours. The organic layer was dried over anhydrous sodium sulphate, concentrated under reduced pressure to obtain a yellowish residue. The partially methylated product thus obtained was next subjected to methylation with Purdie reagent. The compound (5.32 g) was dissolved in dry methanol (55 ml) and mixed with methyl iodide (46 ml). The mixture was refluxed gently and freshly prepared silver oxide (20.2 g) was added to it in small portions during 7 hours. The mixture was filtered and the residue extracted exhaustively with chloroform. The combined filtrate was concentrated to syrup which was repeatedly methylated (four times) with same quantity of reagents as used earlier. The fully methylated polysaccharide was extracted with chloroform, filtered and the solution was evaporated

to a syrup (yield, 3.87 g), $[\alpha]_D^{28}$ + 62.05° (C, 0.8 in chloroform). IR spectrum (Fig. 1.9) of the methylated compound in dry chloroform showed disappearance of characteristic absorption peak at 3400 cm^{-1} due to OH groups.

Hydrolysis of the Fully Methylated Polysaccharide :

Fully methylated polysaccharide (2.5 g) was mixed with sulphuric acid (72% W/W, 45 ml) at 0°C and kept for one hour when the solution turned violet. The strength of the acid was then reduced to 12% (W/W) by the addition of calculated amount of water (225 ml). At this stage, the colour of the solution changed from violet to brownish red. The resulting mixture was heated on a steam bath for 6 hours. After the reaction was over, the solution was cooled, neutralised carefully, filtered and finally concentrated to a syrup (1.64 g) comprising of a mixture of neutral methylated sugars.

Examination of the Neutral Methylated Sugars Mixture :

The separation and characterisation of neutral methylated sugars was performed by preparative partition chromatography and g.l.c.- m.s. technique. The preliminary paper chromatographic examination of the mixture of neutral methylated sugars (solvent S_1, spray reagent R_3) revealed two sharp spots having R_{TMG} values 0.87 to 0.54 along with three faint spots having R_{TMG} values 0.96, 0.80 and 0.71. To examine properly the existence of various methylated sugars, the alditol acetates of neutral methylated sugar mixture was subjected to g.l.c.-m.s. analysis under column condition C_2 as described earlier. The g.l.c. part is given in Fig. 1.11 and the m/z values of the fragmentation patterns as concluded from the mass spectra of various partially methylated sugars are given in Table 1.7. The results

as recorded in Table 1.17 revealed that the sample was composed of five components of the partially methylated sugars.

Resolution of Neutral Methylated Sugar Mixture by Preparative Partition Chromatography and Characterisation of the Components by Classical Methods :

The resolution of two methylated sugars having two sharp spots on paper chromatogram, was carried out by preparative partition chromatography on Whatman No. 3 MM filter paper sheets (solvent S_1 and spray reagent R_3). The chromatogram corresponding to individual sugars were cut and extracted with water according to Dent's procedure[14]. Thus two methylated sugar fractions (A_1 to A_2) were obtained in a homogeneous state, which were characterised as follows.

Characterization of Fraction A_1 :

Syrup (0.2558 g), $[\alpha]_D^{25}$ + 112.2° (c, 1.05 in water) was paper chromatographically indistinguishable (R_{TMG}, 0.87 in solvent S_1) from an authentic sample of 2,3,4,6-tetra-O-methyl-D-galactose (Found : OMe, 52.21; $C_{10}H_{20}O_6$, tetra-O-methyl-D-galactose, requires : OMe, 52.5 percent). Upon demethylation, the compound furnished a spot corresponding to galactose only (R_f, 0.07 in solvent S_1). Finally, the compound was characterised by preparing its 2,3,4,6-tetra-O-Methyl-N-phenyl-D-galactosylamine, m.p.191-92°C (Lit.[26], m.p. 192-93°C).

Characterization of Fraction A_2 :

Syrup (0.2346 g), $[\alpha]_D^{25}$ - 15.7° (C, 0.9 in water), on demethylation furnished mannose only which was identified by paper chromatographic examination (R_f, 0.11 in solvent S_1). It had the same mobility (R_{TMG}, 0.54

in solvent S_1) on paper chromatogram as an authentic sample of 2,3-di-O-methyl-D-mannose (Found : OMe, 29.3; $C_8H_{16}O_6$, Di-O-methyl-D-mannose, requires : OMe, 29.8 percent). Its identity was finally established by its conversion to nitrobenzoate derivative.

The syrup (37.8 mg) was dissolved in dry pyridine (4 ml) and to it was added p-nitrobenzyol chloride (100 mg) while shaking. The solution was heated at 60-70°C for 1 hour and left overnight at room temperature. A saturated solution of sodium bicarbonate was added to the reaction mixture till the effervescence had ceased. A little amount of water added and the product was extracted with chloroform thrice. After drying the chloroform extract with anhydrous sodium sulphate, the solvent was evaporated and the solid obtained was recrystallised from methanol. The 1,4,6-tri-p-nitro-benzoyl-2,3-di-O-methyl-D-mannose had m.p. 193-194°C (Lit.[27], m.p. 192-94°C).

Resolution and Characterisation of Neutral Methylated Sugars by g.l.c.-m.s. Analysis of its Alditol Acetate Derivative :

The syrup of neutral methylated sugar mixture was converted into alditol acetate according to the procedure described earlier[13]. The residue was dissolved in chloroform-methanol mixture (1:1) and applied to g.l.c.-m.s. under the condition C_2 as desdcribed earlier. From the relative retention times (with reference to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol) in g.l.c. chromatogram (Fig.1.10) and the mass fragmentation pattern of individual partially methylated sugars (Table 1.7), it was concluded that the neutral methylated sugar mixture was composed of 2,3,4,6-tetra-O-methyl-mannose, 2,3,4,6-tetra-O-methyl-galactose, 2,3,6-tri-O-methyl-mannose, 2,3,6-tri-O-methyl-galactose. and 2,3-di-O-methyl-mannose.

Quantitative Estimation of Neutral Methylated Sugars Present in the Mixture :

Gas-Chromatographic Method :

The g.l.c. chromatogram of the alditol acetates of neutral methylated sugar mixture has been shown in Fig. 1.11. The results as summarised in Table 1.7 show that 2,3,4,6-tetra-O-methyl-mannose, 2,3,4,6-tetra-O-methyl-galactose, 2,3,6-tri-O-methyl-mannose, 2,3,6-tri-O-methyl-galactose, and 2,3-di-O-methyl-mannose are in molar ratio of 1:40.7:3.2:2.7:42.2 respectively.

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