PART I. STRUCTURAL INVESTIGATION OF TAMARIND KERNEL POLYSACCHARIDE

INTRODUCTION
Tamarindus indica is one of the most common Indian trees which when fully grown can go as high as 80 ft. with a circumference of about 25 ft. The tree flowers in spring and the fruit is ripened in winter. The fruit is a large flat pod and is composed of seed, fibrous matter and an acidic pulp.\(^1\)

The seeds are hard and have a deep brown testa which encloses a creamy white kernel. The seeds had hardly any use until 1942 when it was discovered that the powdered kernel was an excellent sizing material for cotton warp and jute fibres. During the last war, due to the scarcity of food in India, maize was not available for the manufacture of starch. It became therefore imperative to find a suitable substitute for starches for sizing. Tamarind kernel powder is now extensively used for cotton warp sizing and has completely replaced starch as a size for jute.

It is estimated\(^2\) that the total production of tamarind seeds in India would be about 132,000 tons which would yield 90,000 tons of the kernel powder.

Tamarind kernel powder (T.K.Pr.) contains carbohydrates including free sugars, proteins, fat, fibres, inorganic salts and tannins.\(^3,4\) T.K.Pr. disperses in cold or hot water to give a viscous paste. The paste viscosity of T.K.Pr. is much higher than
that of a paste of maize starch of the same concentration. Thus, while a 0.5% T.K.Pr. paste in water at 35° has a relative viscosity of 5.0, a 1.5% paste of maize starch has the relative viscosity of only 4.6. Like maize starch, the pastes of T.K.Pr. thin down with continued heating. However, in contradistinction with the starch pastes, the pastes from T.K.Pr. do not thin down due to the shearing action of the stirrer nor do they retrograde on standing.

T.K.Pr. is thus an important raw material used in the processing of cotton and jute fibres and its availability in large amounts warrants detailed studies on the composition and structure of its carbohydrate polymer component which imparts viscosity to T.K.Pr. pastes. It should also present interesting possibilities of chemical modification to suit particular end uses. For the latter type of studies, it is essential to know the detailed chemical and fine structure of the polysaccharide of tamarind kernel.

TAMARIND KERNEL POLYSACCHARIDE

The major part of the tamarind seed kernel is composed of a water soluble polysaccharide which forms thick mucilaginous solutions. The polysaccharide forms gels in presence of high concentrations of sucrose like fruit pectins and with sodium tetraborate. The polysaccharide, which was originally thought to be a pectin, differs from it in several respects. Thus, the polysaccharide forms a gel with sodium tetraborate, does not contain any uronic acid or methyl-
wronate groups and is not precipitated as calcium salt when treated
The polysaccharide was thought to be a mucilage by some workers because of its origin and its property of forming mucilagenous solutions with water. This classification is not satisfactory in the sense that no neutral mucilage is known to form sugar jellies. Moreover, the polysaccharide contains mostly glucose and xylose residues, sugars which are rarely found in neutral mucilages.

Classification of carbohydrate polymers on the basis of their origin or their physical properties becomes difficult due to the presence of a large variety of polysaccharides in the same part of different plants and because of polysaccharides of different chemical compositions having similar physical properties. Thus, while the seeds of maize, tamarind and guar have polysaccharides of entirely different chemical structures, their physical properties especially from tamarind seeds and guar have definite resemblance. A better method of classification would be to designate polysaccharides according to their component sugars irrespective of their source and physical properties. It is well-known that the physical properties of polymers are dependent on orientation, geometry and three-dimensional arrangement of the molecules.

The polysaccharide of tamarind seed was discovered by Schleiden and Vogel and Schleiden. These authors called it as Tamarindus amyloid because it gave a blue coloration with iodine. This iodine reaction led Rajnarain and Dutt to believe that
The iodine stained section of tamarind kernels, when examined under a microscope, shows that the polysaccharide is present only in the cell walls and not in the cells. \(^{13,14}\)

Different names have been given to the polysaccharide of tamarind kernel such as jellose, polyose, tamarindus amyloid, tikernose by different workers but in this dissertation the polysaccharide will be referred to as Tamarind Kernel Polysaccharide or in short T.K.P.

Krishna and Ghose\(^ {15}\) were the first to study its composition and found that the polysaccharide was composed of glucose, galactose and xylose in a molar ratio of 5:1:2. These findings were later confirmed by Savur and Sreenivasan.\(^ {16}\)

Rao and Beri\(^ {17}\) isolated a hexasaccharide by degradation of the polysaccharide with a mould, Cladosporium herbarum (Fars) link. The hexasaccharide was shown to be composed of glucose, galactose and xylose, the molar ratio of the sugars being 5:1:2. The authors named the oligosaccharide as "tamarindose". It showed \(\nu_\alpha D + 75.8^\circ (H_2O)\) and had a molecular weight of 975 (calculated value for a hexasaccharide = 930) as determined by the cyanide method. The acetyl derivative had m.p. 172\(^\circ\)-174\(^\circ\) (decomp.) and \(\nu_\alpha D + 45.6^\circ\) in methanol. On the basis of these studies, the authors suggested that the hexasaccharide was a repeating unit of the parent polysaccharide.
Detailed studies on the structure of T.K.P. were reported for the first time by White and Rao. The polysaccharide, obtained by repeated precipitations with ethanol, was methylated seven times with methyl sulfate and alkali according to Haworth procedure to yield a methylated polysaccharide having a methoxyl value of 42.5%. The product on methanolysis and subsequent distillation in vacuo afforded methyl glycosides of 2,3,4-tri-O-methyl-β-xylose, 3,4-di-O-methyl-β-xylose, 2,3,4,6-tetra-O-methyl-β-galactose, 2,5,6-tri-O-methyl-β-glucose, and 2,3-di-O-methyl-β-glucose. For determination of the ratios of methylated sugars the polysaccharide methyl ether was hydrolyzed with aqueous sulfuric acid and the methylated sugars estimated by the hypoiodite method. The following ratios of the methylated fragments were found: 2,3,4-tri-O-methyl-β-xylose (1.06 parts); 3,4-di-O-methyl-β-xylose (1.07 parts); 2,3,4,6-tetra-O-methyl-β-galactose (1.82 parts); 2,3,6-tri-O-methyl-β-glucose (0.86 parts) and 2,5-di-O-methyl-β-glucose (1.96 parts). On the basis of these results, the authors proposed structure I (Fig.1) for the repeating unit of T.K.P.

According to this structure, therefore, the polysaccharide has a backbone of 1,4-linked β-glucose residues, every second and third glucose residues of which carry through C6 a side chain of either a galactose residue or a 1,2-linked xylobiose unit. The authors also pointed out the possibility of the glucose residues being in the side chain. However, no definite conclusions could be drawn due to lack of adequate data on the sequential distribution of sugar residues in the polymer.
DIFFERENT STRUCTURES PROPOSED FOR T.K.P. BY DIFFERENT WORKERS:

I. BY RAO & WHITE

\[
\begin{array}{c}
\beta 1^4 \beta 1^4 \beta 1^4 \\
\alpha 6 \alpha 6 \alpha 6 \\
\beta 2 \beta 2 \beta 2 \\
\alpha 1 \alpha 1 \alpha 1 \\
\end{array}
\]

II. BY KHAN & MUKHERJEE

\[
\begin{array}{c}
\beta 1^4 \beta 1^4 \beta 1^4 \beta 1^4 \\
\alpha 6 \alpha 6 \alpha 6 \alpha 6 \\
\beta 1^2 \beta 1^2 \beta 1^2 \\
\alpha 1 \alpha 1 \alpha 1 \\
\end{array}
\]

III. BY KOOLMAN

\[
\begin{array}{c}
\beta 1^4 \beta 1^4 \beta 1^4 \beta 1^4 \\
\alpha 6 \alpha 6 \alpha 6 \alpha 6 \\
\beta 1^2 \beta 1^2 \beta 1^2 \\
\alpha 1 \alpha 1 \alpha 1 \\
\end{array}
\]

G = D - GLUCOPYRANOSE
Ga = D - GALACTOPYRANOSE
X = D - XYLOPYRANOSE

FIG. 1.
Without reference to, but in close agreement with the results of White and Rao, Khan and Mukherjee published data on methylation of T.K.P. The polysaccharide, obtained by repeated precipitations with ethanol, was methylated and the methylated product hydrolysed to afford 2,3,4,6-tetra-\(\beta\)-methyl-\(\beta\)-galactose (1.03), 2,3,4-tri-\(\beta\)-methyl-\(\beta\)-xylose (1.12), 3,4,di-\(\beta\)-methyl-\(\beta\)-xylose (1.11), 2,3-di-\(\beta\)-methyl-\(\beta\)-glucose (1.89) and 2,3,6-tri-\(\beta\)-methyl-\(\beta\)-glucose (1.01 parts). On the basis of these results, they proposed a structure II (Fig.1) identical with one of the structures of the polysaccharide proposed by White and Rao. Khan and Mukherjee, based entirely on the infrared spectral analysis, concluded that the polysaccharide contained only \(\beta\) -linkages.

Savar on the other hand, has claimed to have isolated a xylan from tamarind kernel powder. The crude material was fractionated into three fractions: Fraction A, soluble in water within 2 to 3 minutes at 5°C (yield 2-4%); fraction B, soluble in water at room temperature after 35 minutes stirring (yield 20-22%) and fraction C which consisted of material insoluble in cold but soluble in boiling water (yield 30-35%). Extraction of C with 4% aqueous sodium hydroxide at room temperature, followed by acidification with acetic acid and precipitation with acetone afforded a product which upon fractionation via copper complexing yielded a xylan. The purified xylan was methylated and the methyl ether derivative hydrolysed to produce 2,3,4-tri-\(\beta\)-methyl-\(\beta\)-xylose, 2,3-di-\(\beta\)-methyl-\(\beta\)-xylose and 2-\(\beta\)-methyl-\(\beta\)-xylose with traces of xylose.
No 3,4-di-O-®ethyl-D-xylose was found. On the basis of these results, Savur concluded that the polysaccharide was a xylan composed of $80 \pm 5 \beta$-D-xylopyranose units linked through $1\rightarrow 4$ glycosidic bonds disposed in a singly branched structure, the side chains occurring at C$_3$ of one of the xylopyanosyl units. The reducing power determined by means of the alkaline-iodine method and periodate oxidation studies was reported to support this structure.

Such a wide discrepancy in the structure of T.K.P. arising from the work of different research workers prompted us to undertake a detailed study of the polysaccharide. While this study was in progress Kooinman published results on partial degradation of T.K.P. employing methods of enzymic and acid hydrolysis and acetylation. The polysaccharide, which after two fractionations via copper complexing was shown to be homogeneous, was hydrolysed to produce glucose, xylose and galactose in the ratio of 4:3:1. No other sugars were detected. Partial acid hydrolysis of the polysaccharide with $0.4\text{M} \text{H}_2\text{SO}_4$ in a boiling water bath for 3 hours yielded a degraded polymer composed of glucose and small amounts of xylose. This product showed an X-ray diagram similar to those of cellohexaose and of cellulose II, which indicated that the main chain of T.K.P. consisted of $\beta$-1,4-linked glucose residues. This degraded polymer, on treatment with heated Myrothecium cellulase, produced cellobiose, further proving that it was a $\beta$-1,4-linked glucan.
Kooiman (loc.cit) hydrolyzed T.K.P. with an enzyme preparation "Luzym" which contained among other enzymes a powerful $\beta$-galactosidase, a cellulase and a $\beta$-glucosidase capable of splitting the glucosidic linkages between side chain bearing glucose residues but having no $\alpha$-xlosidase activity. The products of hydrolysis were galactose, glucose and $6-O-\alpha-D$-xylpyranosyl-$D$-glucose.

The degradation of T.K.P. by Myrothecium cellulase yielded a mixture of oligosaccharides, the major components being a heptaose (composed of glucose and xylose - 4:3), an octaose (composed of glucose:xylose:galactose - 4:3:1) and a nonaose (composed of glucose:xylose:galactose - 4:3:2). The oligosaccharides were not characterized adequately. Thus, no information was gathered on the sequential distribution of sugar residues or on the inter sugar linkages present in the oligosaccharides.

Partial acid hydrolysis of the polysaccharide with 0.01M sulfuric acid on a boiling water bath for 146 hours produced 2-0-$\beta-D$-galactopyranosyl-$D$-xylopyranose.

On the basis of the above degradative studies Kooiman proposed structure III (Fig.1) for the repeating unit of T.K.P. No methylation analysis of the polysaccharide was carried out by Kooiman.
RESULTS
The crude polysaccharide obtained by hot water extraction of the defatted tamarind kernels, was purified by complexing with Fehling solution or barium hydroxide solution. The polysaccharide prepared by either of the fractionation procedures was homogeneous as shown by ultracentrifugation and zone electrophoresis. The polysaccharide had $[\alpha]_D +75^\circ$ in water and upon acid hydrolysis produced $D$-glucose, $D$-xylose, $D$-galactose and $L$-arabinose in the molar ratio of $8:4:2:1$ as determined by the phenol-sulphuric acid method of Smith et al.22 The molar ratio of the sugars was also estimated by another method. Fully methylated T.K.P. (see p. 35 for details of preparation) was hydrolysed with acid and the resultant methylated fragments further methylated with methyl iodide and barium oxide-barium hydroxide using $N,N'$-dimethyl formamide (DMF) as solvent according to the procedure of Kuhn.23 Gas chromatographic examination of the product showed the presence of fully methylated methyl glycosides of $D$-glucopyranose, $D$-xylopyranose, $D$-galactopyranose, $L$-arabinofuranose and $L$-arabinopyranose (present only in trace amounts). The ratio of $D$-glucose : $D$-xylose : $D$-galactose : $L$-arabinose, as estimated by the peak areas of their respective fully methylated methyl glycosides, was found to be $8.5 : 4.2 : 2 : 1$. These values are in close agreement with the ratios of the sugars obtained by the phenol-sulfuric acid method.

Partial hydrolysis of the polysaccharide with $0.4N$ sulfuric acid
Disaccharides obtained on acetylation of TKP.

GLUCOSE → HYDROLYSIS → ACETIC ANHYDRIDE → CELLOBIOSE OCTAACETATE

CH₃COONa

GALACTOSE → HYDROLYSIS → 1) REDUCTION → GALACTOSE

2) HYDROLYSIS → 1) METHYLATION → 2,3,4,6-TETRA-O-METHYL-D-GALACTOSE

+ 3,4-DI-O-METHYL-D-XYLOSE

2-O-β-D-GALACTOPYRANOSYL-D-XYLOPYRANOSE

CH₂OH

CH₂OH

CELLOBIOSE

CELLOBIOSE

H₂O
DISACCHARIDES OBTAINED ON ENZYMIC HYDROLYSIS OF T. K. P.

III
LACTOSE

HYDROLYSIS GLUCOSE + GALACTOSE
(1) REDUCTION GALACTOSE
(2) HYDROLYSIS 2,3,4,6- TETRA-O-METHYL-D-GALACTOSE
(2) HYDROLYSIS 2,3,6- TRI-O-METHYL-D-GLUCOSE

PHENYLHYDRAZINE LACTOSAZONE

HYDROLYSIS GLUCOSE + XYLOSE
(1) REDUCTION XYLOSE
(2) HYDROLYSIS 2,3,4- TRI-O-METHYL-D-GLUCOSE
(2) HYDROLYSIS 2,3,4- TRI-O-METHYL-D-XYLOSE

IV
6-O-\(\alpha\)-D-XYLOPYRANOSYL-D-GLUCOPYRANOSE

FIG. 2b
galactose, showing thereby that the reducing end of the disaccharide was constituted by the xylose residue. The disaccharide gave a negative colour reaction with triphenyl tetrazolium chloride indicating a 1→2 inter-sugar linkage. Methylation of the disaccharide followed by acid hydrolysis of the fully methylated derivative afforded 2,3,4,6-tetra-O-methyl-D-galactose and 3,4-di-O-methyl-D-xylose in equal proportions. These results proved that oligosaccharide B was 2-O-D-galactopyranosyl-D-xylopyranose. The low positive rotation (+52°) of the sugar indicated that the bise linkage was of the β type.

Enzymic hydrolysis of the polysaccharide with an enzyme preparation "hemicellulase", produced two oligosaccharides C and D in addition to glucose, xylose, arabinose and traces of galactose. Oligosaccharides C and D were obtained in yields of 14% and 40% respectively on the weight of dry polysaccharide.

Oligosaccharide C was identified as 4-O-β-D-galactopyranosyl-D-glucose (Fig.2b III, Lactose) in the following manner. Acid hydrolysis of C produced galactose and glucose in equimolar proportions. The degree of polymerisation, as determined by Timell's method was found to be 1.78. An authentic sample of lactose, by the same method, gave a value of 1.8. The oligosaccharide C was therefore a disaccharide composed of galactose and glucose residues. Reduction of the disaccharide with sodium borohydride followed by acid hydrolysis of the polyol gave only one reducing sugar, namely, galactose, indicating thereby that the disaccharide
was a galactosyl glucose. The disaccharide gave a positive color reaction with triphenyl tetrazolium chloride thus showing the absence of a $1 \rightarrow 2$ linkage. When treated with aniline-diphenylamine phosphate reagent it produced a greenish blue colour indicating the possibility of a $1 \rightarrow 4$ linkage. An authentic sample of lactose by this reagent produced a similar color. The disaccharide crystallised from aqueous ethanol to provide triangle-shaped crystals which had m.p. 200–201°C and $\alpha$-D $+60.5^\circ$ in water. The disaccharide was methylated and hydrolysis of the methyl ether produced equimolar proportions of 2,5,4,6-tetra-$\alpha$-methyl-$D$-galactose and 2,3,6-tri-$\alpha$-methyl-$D$-glucose, showing that the disaccharide was 4-$\alpha$-$D$-galactopyranosyl-$D$-glucose. The identification of the disaccharide as lactose was further confirmed by examination of its infrared spectrum and preparation of the characteristic phenylsazone derivative.

The identification of oligosaccharide $D$ as 6-$\alpha$-$D$-xylopyranosyl-$D$-glucopyranose (Fig. 2b, IV) was based upon the following experimental evidence. The sugar was a disaccharide as shown by the determination of D.P. by Timell's method. Acid hydrolysis of the disaccharide produce xylose and glucose in equimolar proportions. Reduction of the disaccharide with sodium borohydride followed by hydrolysis of the resulting product gave only one reducing sugar, namely, xylose showing thereby that the disaccharide was a xylosyl glucose. A $1 \rightarrow 2$ linkage was unlikely inasmuch as the disaccharide gave a positive color
reaction with triphenyl tetrazolium chloride. Methylation of the disaccharide according to Kuhn's procedure followed by acid hydrolysis of the methyl ether derivative produced 2,3,4-tri-O-methyl-D-glucose and 2,3,4-tri-O-methyl-D-xylose in equal proportions. The disaccharide was, therefore, 6-O-D-xylopyranosyl-D-glucopyranose, the high positive rotation $[\alpha]^D_{D+120^\circ}$ in water of the sugar indicating an $\alpha$ inter-sugar linkage.

The polysaccharide could not be methylated completely by the usual methods of methylation by reagents such as dimethyl sulfate and alkali (Haworth), methyl iodide and silver oxide (Purdie) and by liquid ammonia, metallic sodium and methyl iodide. Complete methylation was however, achieved by the DMSO method developed in this laboratory. According to this method, the polysaccharide was dissolved in dimethyl sulfoxide (DMSO) and the methylation carried out by dimethyl sulfate and solid sodium hydroxide. The methylated polysaccharide which had a methoxyl value of 43.0% and $[\alpha]^D_{D+50^\circ}$ (in CHCl₃) was fractionated with diethyl ether and petroleum ether (b.p.40-60°C). The fractionated methylated polysaccharide was hydrolyzed by the method of Croon et al. and the methylated fragments separated by paper and cellulose column chromatography. The following methylated sugars which were identified by gas chromatography and by way of crystalline derivatives, were obtained in molar ratios mentioned against each in parentheses.

2,3,5-Tri-O-methyl-L-arabinose (1), 2,3,4-tri-O-methyl-D-xylose (5), 2,3,4,6-tetra-O-methyl-D-galactose (2), 5,4-dimethyl-D-xylose (1),
2,3,6-tri-O-methyl-D-glucose (2) and 2,3-di-O-methyl-D-glucose (6). The mixture of 3,4-di-O-methyl-D-xylose and 2,3,6-tri-O-methyl-D-glucose, which could not be resolved by paper chromatography in several solvent systems, was readily separated into its components by paper electrophoresis in borate buffer.

The polysaccharide was oxidised with 0.05 M sodium metaperiodate at 10° when 1.55 moles of the oxidant was consumed and 0.54 mole of formic acid was liberated per mole of anhydro sugar unit (152, calculated on the basis of a polymer composed of glucose, xylose, galactose and arabino in the ratio of 8:4:2:1). The oxopolysaccharide, upon reduction with sodium borohydride and subsequent hydrolysis with acid, produced glycerol, erythritol and glyceraldehyde in the molar ratio of 2.5:8:1. Estimation of glycerol in presence of glyceraldehyde by the usual periodate oxidation-chromotropic acid procedure is complicated by the same Rf value of the two substances on paper. This difficulty was resolved by removal of glyceraldehyde from the mixture by bromine oxidation followed by absorption on anion exchange resin. The amount of glyceraldehyde in the mixture was computed by difference in the values of glycerol obtained (1) as above where the glyceraldehyde component was eliminated by oxidation and (2) by reduction of the glyceraldehyde to glycerol. No sugar residues in the polymer survived periodate oxidation as shown by the absence of sugars in the hydrolysate of periodate oxidized and borohydride reduced polysaccharide.
DISCUSSION
DISCUSSION

The polysaccharide on hydrolysis produced glucose, xylose, galactose and arabinose in the molar ratio of 8:4:2:1. It is rather surprising that in the two detailed investigations carried out on the polysaccharide by White and Rao and by Kooiman no mention is made of the presence of arabinose. Consequently, in their formulations of T.K.P., arabinose is not shown as a constituent. In our studies, arabinose was easily detected by partial and complete hydrolysis of the polysaccharide as well as by the hydrolysis of its methyl ether. In fact, in the partial hydrolysis of the polysaccharide by 0.01N sulfuric acid, arabinose is released first as shown by paper chromatographic analysis. That arabinose is present in the \( \text{\(L\)}\)-arabino furanose form follows from the identification of 2,3,5-tri-O-methyl-\( \text{\(L\)}\)-arabinose in the hydrolysate of methylated T.K.P.

It is not likely that the arabinose detected by us arose from an associated araban, inasmuch as the polysaccharide was shown to be homogeneous both by the sedimentation method and by ionophoresis. In the literature there have been conflicting reports from time to time about the presence of arabinose in the polysaccharide. However, in these investigations the homogeneity of the polymer was not established nor was a detailed structure of T.K.P. undertaken. Recently Macmillan et al. in conformity with our results, have reported that T.K.P. contains arabinose residues besides the sugars glucose, xylose and galactose. These authors carried out a detailed
study on the fractionation of T.K.P. and have shown that even after fractionation of the polysaccharide according to the method of Savur\textsuperscript{20} the fractionated polymer on hydrolysis afforded glucose, xylose, galactose and arabinose. Apart from the presence of arabinose in the T.K.P. hydrolysate, the ratio of the sugars glucose, xylose and galactose as found in the present investigation (4:2:1) is different from that found by White and Rae\textsuperscript{18} (5:2:1) and by Kooiman\textsuperscript{21} (4:5:1.33). Rae’s polysaccharide preparation was only ethanol fractionated and had not undergone any extensive fractionation procedure. The homogeneity of the polysaccharide was also not known. So far as Kooiman’s polysaccharide is concerned, although it was well fractionated and found to be homogeneous, the sugars were determined in the hydrolysate obtained by heating the polymer with 1\textdegree M sulfuric acid for 4 hours. Under these conditions, it is likely that the polysaccharide was not hydrolyzed completely to sugars. Graded hydrolysis of T.K.P. has shown that the backbone of glucose residues is most resistant to hydrolysis. The relatively mild conditions of hydrolysis employed by Kooiman may not have hydrolyzed this backbone completely, thus resulting in a lower ratio for glucose.

That the polysaccharide has a backbone of \textgreek{D}-glucose residues linked through 1\textsuperscript{→}4 linkages, emerges from the methylation data of White and Rao\textsuperscript{18} and from the acetylation and partial acid hydrolysis experiments of Kooiman.\textsuperscript{21} This is supported by our studies on the acetylation, partial hydrolysis and methylation analysis of the
polysaccharide. Thus, cellobiose was obtained on acetolysis and a relatively resistant core of glucosan resulted on partial hydrolysis.

The mole ratios of methylated fragments, produced on hydrolysis of T.K.P. methyl ether, as obtained by different workers are given in Table I.

**TABLE I**

**METHYLATED SUGARS (IN MOLES) FROM T.K.P. METHYL ETHER**

<table>
<thead>
<tr>
<th>Methylated sugar</th>
<th>White and Rao(^{18})</th>
<th>Khan and Mukherjee(^{19})</th>
<th>Calculated from the structure proposed by Kooiman(^{21})</th>
<th>Results of present study</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,4-Tri-O-methyl-D-xylose</td>
<td>1.06</td>
<td>1.12</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>3,4-Di-O-methyl-D-xylose</td>
<td>1.07</td>
<td>1.1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2,3,4,6-Tetra-O-methyl-D-galactose</td>
<td>1.02</td>
<td>1.05</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>2,3,6-Tri-O-methyl-D-glucopyranose</td>
<td>0.86</td>
<td>1.01</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>2,3,Di-O-methyl-D-glucopyranose</td>
<td>1.96</td>
<td>1.89</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>2,3,5-Tri-O-methyl-L-arabinose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
</tbody>
</table>

It will be observed that White and Rao\(^{18}\) and Khan and Mukherjee\(^{19}\) report the same molar ratios of methyl ethers of glucose, xylose and galactose. If one calculates the molar ratios of
methylated sugars which a polysaccharide of the structure proposed by Kooiman would give; it is found that these are very different from those obtained by the previous two groups of workers. The main difference is that in the Kooiman structure the ratio of tri-\(\beta\)-methyl xylene to di-\(\beta\)-methyl xylene is two and that of di-\(\beta\)-methyl glucose to tri-\(\beta\)-methyl glucose three as against the corresponding ratios of 1 and 2 found by White and Rao and Khan and Mukherjee. Our results in respect of methyl ethers of glucose, xylose and galactose are in agreement with the proposed structure of Kooiman except for higher molar ratio of the tri-\(\beta\)-methyl xylene to di-\(\beta\)-methyl xylene. The frequency of branched xylose units across the molecular chain would thus appear to be less than that shown in the Kooiman structure. Furthermore, our findings are different from that of all the previous workers in so far as the isolation of 2,3,5-tri-\(\beta\)-methyl-\(L\)-arabinose is concerned.

The general picture which emerges from the methylation analysis, is that of a highly branched polymer in which galactose, xylose and arabinose residues constitute the non-reducing ends with a backbone of glucose residues. The highly branched character of the polysaccharide probably explains its resistance towards complete methylation by the usual alkali decimation procedures.

The isolation of 6-\(\beta\)-\(\alpha\)-xylopyranosyl glucose (Fig.2b, IV)
and 2-Ω-β-D-galactopyranosyl-D-xylopyranose (Fig.2a, II) by enzymic hydrolysis and acetolysis respectively, indicates that some of the xylose residues and the disaccharide II constitute a portion of the branches in the polymer. These results corroborate the work of Kooiman\textsuperscript{21} on controlled degradation of T.K.P.

Hydrolysis of the polysaccharide with the enzyme preparation "hemicellulase" produced, in good amounts, 4-Ω-β-D-galactopyranosyl-D-glucose (Lactose). This showed that in certain parts of the molecule, galactose and glucose residues were directly connected with each other through β 1→ 4 glycosidic bonds. The lactose unit can be fitted into the polymer structure either by envisaging some 1→ 6 linkages in the backbone of 1→ 4 glucose residues (structure A) or by postulating that some of the glucose units occur in branches (structure B). The latter possibility was mentioned by White and Rao\textsuperscript{18} but was discounted by Kooiman.\textsuperscript{21}

![Diagram of structures A and B](image)

Supporting evidence in favour of structure B has been gathered in the following manner. The resistant core of T.K.P., a glucosan, obtained by hydrolysis of T.K.P. with 0.4 M sulfuric acid for 3 hours,
was methylated and the methylated polymer hydrolysed. In the mixture of methylated fragments, no 2,3,4-tri-O-methyl-D-glucose was detected by paper chromatographic analysis. Since in the glucosan there was no galactose present, the Gal₁→₄Gal bond must have hydrolysed during acid hydrolysis of T.K.P. However, a Gal₁→₆Gal linkage will survive inasmuch as this linkage is more stable than the 1→4 glucosidic bond as shown by Wolfrom. Therefore, after partial degradation of the polymer, structures C and D will result from the partial structures A and B. Methylation and hydrolysis of a polymer of structure C should yield 2,5,4-tri-O-methyl-D-glucose in addition to other methylated sugars whereas a similar treatment to polymer of structure D will not result in any 2,5,4-tri-O-methyl-D-glucose.

\[
\begin{align*}
\text{Structure C} & & \text{Structure D} \\
4.1 & 6.1 & 4.1 & 4.1 & 4.1 & 4.1 & 6 & 1 \\
\end{align*}
\]

Enzymic hydrolysis of T.K.P., produced lactose and 6-O-\(\alpha\)-D-xylopyranosyl-glucopyranose in yields of 14% and 40% respectively. If one lactose unit were present per repeating unit of the polysaccharide composed of glucose, xylose, galactose and arabinose (8:4:2:1), the theoretical yield of this disaccharide would have been 15%. The isolation of lactose in near theoretical yield therefore shows that in the repeating unit of the polymer one such unit is present. The
isolation of 2-\(\alpha\)-\(\beta\)-galactopyranosyl-\(\beta\)-xylose by the acetolysis of T.K.P. shows that in the polymer certain of the galactose residues are linked to xylose at \(C_2\). Since the methylation analysis shows that all the galactose units occur as non-reducing ends, the polymer must have in its repeating unit one branch each of 4-\(\alpha\)-\(\beta\)-galactopyranosyl-\(\alpha\)-glucose and 2-\(\alpha\)-\(\beta\)-galactopyranosyl-\(\beta\)-xylose. That all the galactose residues are not linked with xylose residues is also supported by the results of methylation. Thus while the yield of 2,3,4,6-tetra-\(\alpha\)-methyl-\(\alpha\)-galactose was 2 moles, that of 3,4-di-\(\alpha\)-methyl-\(\beta\)-xylose was only one mole. The structure of Kooiman should require equal amounts of the two methylated sugars.

Isolation of 6-\(\alpha\)-\(\alpha\)-xylopyranosyl-\(\alpha\)-glucopyranose by enzymic hydrolysis of T.K.P. in 40% yield shows that the xylose residues are attached to the glucose residues through 1\(\rightarrow\)6 linkages. If all the xylose residues were attached to glucose units in the repeating unit of the polymer, the theoretical yield of 6-\(\alpha\)-\(\alpha\)-xylopyranosyl-\(\alpha\)-glucopyranose should be 55%. The low yield of the disaccharide could be due either to incomplete hydrolysis or to the presence of small amounts of xylosidase in the enzyme preparation resulting in the cleavage of xylose 1\(\rightarrow\)6 glucose bond. In fact, chromatographic analysis of the hydrolyzate did show a spot for xylose.

The position of the arabinofuranose residues with respect to other substituents of the glucose backbone is not known. In order to explain the high molar ratio of 2,3-di-\(\alpha\)-methyl-\(\beta\)-glucose, it could
be assumed that arabinose is linked to the main chain of glucose units through C6. The \( \alpha \) - or \( \beta \) - nature of the linkage is not known.

In the absence of any information on the sequential distribution of substituents along the glucose chain, the above data can be fitted into a number of structures for the repeating unit. Considering that the backbone of the polymer is composed of glucose residues linked only through \( \beta-1 \rightarrow 4 \) bonds, one such structure is represented below:

\[
\begin{array}{cccccc}
\beta & 4 & 1 & \beta & 4 & 1 \\
\beta & 4 & 1 & \beta & 4 & 1 \\
\beta & 4 & 1 & \beta & 4 & 1 \\
\beta & 4 & 1 & \beta & 4 & 1 \\
\beta & 4 & 1 & \beta & 4 & 1 \\
\end{array}
\]

The periodate oxidation data are also in agreement with the above structure proposed for the polysaccharide. Thus, a polysaccharide of this constitution, on periodate oxidation, should consume 1.53 moles of the oxidant with concomitant liberation of 0.53 moles of formic acid. In actual experiment, 1.55 moles of periodate was consumed and 0.54 mole of formic acid was liberated. Consistent with the proposed structure, no sugar residues survived periodate oxidation. Supporting evidence for the structure is also forthcoming from the Smith degradation of the polymer. Thus, the periodate oxidized and reduced polysaccharide afforded, on hydrolysis, glycerol, erythritol and glyceraldehyde in the molar ratio of 2.5:8:1.
The proposed structure of the polymer requires the ratio 5:8:1.

The sequence of reactions leading to the formation of these degradation products is given in Fig. 5.
SMITH DEGRADATION OF T.K.P.

ERYTHRITOL
GLYCEROL
GLYCERALDEHYDE
GLYCOL
GLYCOLALDEHYDE
EXPERIMENTAL
GENERAL METHODS AND TECHNIQUES

(1) EVAPORATION

All evaporations were carried out under reduced pressure and at low temperature (bath temp. 50-55°C).

(2) MELTING POINTS

All melting points are corrected.

(3) OPTICAL ROTATION

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

(4) PAPER CHROMATOGRAPHY

Paper chromatography was carried out by the descending technique on Whatman filter paper Nos. 1 and 3 using the following solvent systems (V/V):

(A) Butan-1-ol-Pyridine-Water (6:4:3).58
(B) Pyridine-Ethylacetate-Water (2:5:5).39
(C) Butanone-Water azeotrope.40
(D) Benzene-Ethanol-Water-Ammonia (200:47:14:1).41

The following reagents were used to detect the sugars on chromatograms:

(a) Acetic acid silver nitrate-alcoholic sodium hydroxide reagent.42
(b) p-Anisidine hydrochloride spray reagent.43
(5) **ZONE ELECTROPHORESIS**

Zone electrophoresis was carried out on Whatman No.1 filter paper strips at 600 Volts for 3 hours in 0.1M sodium tetraborate solution. The sugars on the paper were detected by either of the reagents (a) or (b).

(6) **INFRARED SPECTROSCOPY**

The infrared spectral measurements were carried out on Perkin-Elmer Infracord-137, a double beam instrument. The samples were analyzed in any of the forms mentioned below:

(i) as solutions in chloroform or carbon tetrachloride using 0.1 mm thickness sealed cells.

(ii) as pellets of potassium chloride.

(iii) as mulls in paraffin.

(7) **ACID HYDROLYSIS**

Acid hydrolysis of the samples was carried out in 1M sulfuric acid in sealed tubes at 95°C for 12-16 hours. The hydrolyzates were neutralized with barium carbonate and then filtered. The filtrates, after desionizing with Amberlite IR-120 (H+) and IR-45 (OH) resins, were concentrated for further examination.

(8) **DETERMINATION OF SUGAR RATIOS**

The determination of the ratios of sugars in oligosaccharide
and polysaccharide hydrolyzates was carried out by the Phenol-
sulfuric Acid method of Smith et al. 22

(a) Preparation of Standard Curves for Glucose, Xylose,
Galactose and Arabinose.

Dried sugar (40 mg.) was dissolved in water (1 l.) and
aliquots of the solutions containing 8-48 micrograms of the
sugars were taken in triplicate and the volume adjusted to 2 ml.
with water. Required amounts of 80% aqueous phenol was added to
each solution followed by sulfuric acid (5 ml.) taking care that
the acid fell straight on to the solution. After thorough mixing,
the tubes were allowed to stand for 15 minutes and then cooled in
running water. A water blank for each sugar was also prepared in
the same way and was used to adjust the colorimeter (Spectronic 20)
to zero. The absorbance was read at appropriate wave lengths for
each sugar. The amount of phenol used and the wave lengths at
which the absorbance was read for different sugars is given in
Table II.

TABLE II

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Amount of 80% phenol in ml.</th>
<th>Wave length at which the absorbance is read.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.1</td>
<td>490 nm.</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.04</td>
<td>&quot;</td>
</tr>
<tr>
<td>Xylose</td>
<td>0.02</td>
<td>480 nm.</td>
</tr>
<tr>
<td>Arabinose</td>
<td>0.02</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

26
The absorbance readings for different amounts of sugars are given in Table III and the standard curves in Fig. 4.

**TABLE III**

<table>
<thead>
<tr>
<th>Micrograms of Sugars</th>
<th>Glucose</th>
<th>Galactose</th>
<th>Xylose</th>
<th>Arabinose</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>0.07</td>
<td>0.65</td>
<td>0.115</td>
<td>0.10</td>
</tr>
<tr>
<td>16</td>
<td>0.17</td>
<td>0.14</td>
<td>0.235</td>
<td>0.215</td>
</tr>
<tr>
<td>24</td>
<td>0.26</td>
<td>0.20</td>
<td>0.34</td>
<td>0.315</td>
</tr>
<tr>
<td>32</td>
<td>0.35</td>
<td>0.29</td>
<td>0.44</td>
<td>0.42</td>
</tr>
<tr>
<td>40</td>
<td>0.46</td>
<td>0.34</td>
<td>0.56</td>
<td>0.525</td>
</tr>
<tr>
<td>48</td>
<td>0.52</td>
<td>0.41</td>
<td>0.68</td>
<td>-</td>
</tr>
</tbody>
</table>

(b) Determination of Sugar Ratios in the Polysaccharide and Oligosaccharide.

The different sugars, obtained on hydrolysis of the oligo- or polysaccharide were resolved by paper chromatography using solvent A. The different sugar zones, as revealed by guide strips, were cut out and extracted with equal amounts of water for an hour. The extract was filtered through a glass wool plug and aliquots of the filtrate were taken and the sugar estimated according to the procedure outlined earlier for pure sugar solutions. A portion of the paper chromatogram, having no sugar on it but equal to the area of sugar containing zone, was extracted with water; this extract served as the blank for colorimetric readings.
STANDARD CURVES OF SUGARS

○ XYLOSE
△ ARABINOSE
● GLUCOSE
× GALACTOSE

FIG. 3
(9) **DETERMINATION OF METHOXYL VALUE**

The methoxyl content of samples was determined by the modified Ziesel's method.45

(10) **DEMETHYLATION OF O-METHYLATED SUGARS WITH BORON TRICHLORIDE**46

The methylated sugar (5-10 mg.) was suspended in dichloromethane (2 ml.) and cooled in acetone-solid CO₂ mixture. Boron trichloride (1 ml., cooled in acetone-solid CO₂ mixture) was added to the sugar suspension and kept at -80° for half an hour and then slowly allowed to attain room temperature. The reaction mixture, after keeping for a further 16 hours under anhydrous conditions, was treated with a suspension of silver carbonate in water, the mixture filtered and the filtrate evaporated. The demethylation efficiency was judged by paper chromatographic analysis.

(11) **DETERMINATION OF REDUCING END SUGAR UNIT IN THE OLIGOSACCHARIDES**24

The oligosaccharide (5-10 mg.) in water (2 ml.) was treated with 0.2% solution of sodium borohydride (2 ml.) for 2 hours. The solution was then hydrolyzed with sulfuric acid (2N, 2 ml.) and the sugars produced were detected by paper chromatography in solvents A and B using reagents (a) and (b).

(12) **DETERMINATION OF DEGREE OF POLMERIZATION**26

Oligosaccharide (1 mg.) was dissolved in water (10 ml.) and from the resulting solution eight one ml. aliquots were taken in eight
test tubes. Two test tubes each containing water (1 mL.) served as blanks. Active borohydride solution (0.5 mL. of a 1% solution in water) was added to the first four test tubes and reduction allowed to proceed at room temperature for one hour. After one hour, 2N sulfuric acid (0.5 mL.) was added to the remaining six test tubes (4 sugar solutions and 2 blanks). Aqueous phenol (1.0 mL., 3% in water) followed by conc. sulfuric acid (5 mL.) was added to each of the ten test tubes. After thorough mixing the solutions were allowed to stand for 10 minutes, the tubes, after cooling in water, were kept at room temperature for one hour. Absorbance measurements were carried out on Spectronic 20 (Bausch & Lomb) spectrophotometer at a wave length of 480 nm. The absorbance of the eight sugar solutions (4 reduced and 4 non-reduced) were measured against blanks. The average of the four determinations was used for calculating the D.P.

If $A_1$ is the absorbance of the oligosaccharide hydroxylate before reduction and $A_2$ after reduction, then

$$Q = \frac{A_1}{A_2} \quad \text{and} \quad \text{D.P.} = \frac{Q}{Q-1}$$

where $Q$ = Reducing quotient

D.P. = Degree of polymerization

(13) GAS CHROMATOGRAPHY

Gas-liquid partition chromatography was carried out on a Pye Argon gas chromatograph using four feet glass columns of (1) Apiezon M,
28% on Celite 545, at 150°C., argon flow, 40 ml./minute and

(2) Butanediol succinate polyester, 20% on Celite 545 at 175°C.,
argon flow 60 ml./minute.
PURIFICATION OF THE POLYSACCHARIDE

(A) Fractionation via Copper Complexing

Defatted tamarind kernel powder (10 g.) was extracted with water (1 l.) in a blender. The suspension was then heated with stirring on a boiling water bath for an hour and centrifuged to remove suspended matter (2.2 g.). To the supernatant, Fehling solution (25 ml., diluted to 50 ml. with water) was added dropwise with continuous stirring. The gel-like copper complex was filtered on muslin cloth, washed with dilute Fehling solution and squeezed to remove excess of the reagent. The complex was then suspended in water (500 ml.), cooled in an ice-salt bath, and with stirring acidified with cold 0.2N hydrochloric acid. The resulting solution was poured into ethanol (1.5 l.) to regenerate the polysaccharide. The precipitate was squeezed and then washed with rectified spirit, absolute ethanol, ether and petroleum ether (b.p.40-60°) each three times, in this order, to yield a white fluffy polysaccharide (6 g.); \[\alpha_D^0 = 65.5^0\] in water (c, 0.5). The polysaccharide, obtained as above, was once again fractionated through copper complex formation. The twice-fractionated polysaccharide (4.2 g.) showed \[\alpha_D^0 = 75^0\] in water (c, 0.5).

(B) Fractionation via Barium Complexing

Defatted tamarind kernel powder (10 g.) was extracted with water in a blender and then on a boiling water bath for one hour. The solution was centrifuged to remove insoluble matter (2.5 g.) and to the supernatant saturated barium hydroxide solution (25 ml.) was added.
The barium complex, which separated, was centrifuged. The complex was suspended in water (400 ml.) and after cooling in an ice-salt bath, was decomposed by adding cold 0.2N hydrochloric acid. The resulting solution was poured into ethanol (1 l.) to precipitate the polysaccharide which was then dried by solvent exchange in the usual manner. The polysaccharide (5.5 g.) which had $\alpha_D +58.3^\circ$ in water ($c$, 0.5), was fractionated once more through barium complex formation. The twice-fractionated polysaccharide (3.8 g.) had $\alpha_D +75^\circ$ in water ($c$, 0.5).

**CHARACTERIZATION OF THE POLYSACCHARIDE**

The work reported herein was carried out on the polysaccharide fractionated twice with Fehling solution. For this purpose, 100 g. of the tamarind kernel powder was fractionated to afford 40 g. of the pure polysaccharide.

**Homogeneity of the Polysaccharide**

(a) **Ultracentrifugation**

The polysaccharide samples obtained by the two fractionation procedures were examined in the ultracentrifuge. When a 0.1% solution of the polysaccharide in 0.1M sodium tetraborate solution, was centrifuged at 42,040 r.p.m. in Spinco Model E Analytical Centrifuge, a single symmetrical peak was observed (see Fig.5) indicating thereby that the polysaccharide was homogeneous.
SEDIMENTATION OF PURIFIED TKP.
0.1% IN 0.1 M SODIUM TETRABORATE
ROTOR SPEED 42,040 RPM, TEMP. 20.8°C, TIME 141 MINUTES

TOP PATTERN - TKP. TWICE FRACTIONATED VIA COPPER COMPLEXING
BOTTOM PATTERN - TKP. TWICE FRACTIONATED VIA BARIUM COMPLEXING
PHOTOS TAKEN AFTER, 13; 29; 45; 61; 77; 93; 109; 125; 141 MINUTES

FIG. 4
(b) **Infrared Spectrometry**

The infrared spectra of the two samples taken as KCl discs and as paraffin mulls were found to be identical (see Fig. 6).

(c) **Zone Electrophoresis**

Zone electrophoresis of the polysaccharide sample obtained via copper complexing, was carried out in borate buffer (pH 10). The results are given in Table IV.

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>EMF (Volts)</th>
<th>Current (ma)</th>
<th>Time (hours)</th>
<th>Mg Values*</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>657</td>
<td>12.0</td>
<td>2</td>
<td>0.31</td>
</tr>
<tr>
<td>II</td>
<td>675</td>
<td>15.0</td>
<td>2</td>
<td>0.29</td>
</tr>
<tr>
<td>III</td>
<td>690</td>
<td>15.5</td>
<td>2</td>
<td>0.30</td>
</tr>
</tbody>
</table>

*Mg values represent the rate of movement of the sample with respect to D-glucose.

Acid Hydrolysis

The polysaccharides (100 mg. each), obtained by the two fractionation procedures, were hydrolyzed and the hydrolyzates after working up were examined chromatographically in solvents A and B using reagents (a) and (b). The chromatograms revealed the presence of glucose, galactose, xylose and arabinose.
INFRA-RED SPECTRA OF T.K.P.
A - MULL IN PARAFFIN
B - KCL DISC
Determination of Sugar Ratios

The sugar ratios in the hydrolyzates were determined by the phenol sulfuric acid method. The molar ratios of glucose: xylose: galactose and arabinose in both the samples was found to be 8:4:2:1.
METHYLATION OF T.K.P.

Experiment I, Haworth Methylation: T.K.P. (6.5 g.) was suspended in water (60 ml.) and to the suspension 40% sodium hydroxide (180 ml.) and dimethyl sulfate (60 ml.) were added drop by drop during a period of six hours with constant vigorous stirring. For the first three hours, the reaction was carried out at low temperature (15-20°C) and in an atmosphere of nitrogen. After three hours, the bath temperature was raised to 50°C. The reaction mixture was stirred overnight and then heated at 95°C. for an hour to decompose the excess of dimethyl sulfate. After cooling in an ice-salt bath, the excess of alkali was partially neutralized with cold 10N sulfuric acid and finally with dilute acetic acid. The partially methylated polysaccharide which separated on the surface was removed. The product was taken up in 40% sodium hydroxide and once again methylated by adding dropwise 40% sodium hydroxide (180 ml.) and dimethyl sulfate (60 ml.) at 50°C. Dioxane was added to keep the methylated polysaccharide in solution. After overnight stirring, the reaction mixture was worked up, as described above, to give a partially methylated polysaccharide (5.4 g.) which was insoluble in methanol, acetone and chloroform, but dissolved readily in N,N-dimethyl formamide (DMF).

Kuhn Methylation: The partially methylated T.K.P. (5.4 g.) was dissolved in DMF (80 ml.) and methylation carried out by adding methyl iodide (50 ml.) and barium oxide-barium hydroxide mixture.
in small amounts during a period of six hours with constant stirring according to the method of Kuhn et al. The reaction mixture, after stirring overnight, was poured into chloroform to precipitate the inorganic salts. The chloroform solution was washed successively with thiosulfate solution (to remove iodine) and with water, dried ($\text{Na}_2\text{SO}_4$) and evaporated to yield the methylated polysaccharide 4.5 g., $-\text{OCH}_3$, 52.0%.

**Purdie Methylation:** The above product was dissolved in acetone (80 ml.) and the solution refluxed for 12 hours with methyl iodide (50 ml.) and silver oxide (20 g.), the latter being added in small amounts over a period of 5 hours. The reaction mixture was filtered, the residue washed with chloroform and the filtrate evaporated to a sirup. As the product was soluble in methyl iodide, further Purdie methylations were carried out by dissolving the partially methylated product in methyl iodide and refluxing with silver oxide. Four such methylations yielded a product (4.7 g.) having a methoxyl content of 37.5%. Infrared spectrum of the methylated polysaccharide in chloroform solution showed a prominent hydroxyl peak in the region 3400-3600 cm$^{-1}$ indicating the lack of complete methylation. Further methylations by this method did not increase the methoxyl value.

In order to complete the methylation of the polysaccharide, the partially methylated product from Purdie reactions was dissolved in DMF (60 ml.) and the methylation carried out by Kuhn's procedure.
using silver oxide (20 g.) and methyl iodide (30 ml.). After overnight stirring, the reaction mixture was centrifuged and the supernatant poured into water. Potassium cyanide (50 ml., 2.5% solution) was added to precipitate the silver salts. The mixture was then filtered and the filtrate extracted with chloroform. The chloroform extract was washed successively with thiosulfate solution and with water, dried (Na₂SO₄) and evaporated to yield (3.5 g.) of a light brown friable solid (-OCH₃: 39.5%). The infrared spectrum of the product still showed some hydroxyl absorption.

Experiment II: T.K.P. (5 g.) was methylated three times by the Haworth method and four times by the Purdie method to yield a product (4.2 g.) having a methoxyl content of 37.2%. The infrared spectrum of the substance showed a pronounced hydroxyl peak. Further methylations by Purdie reagents did not increase the methoxyl content.

Experiment III: Methylation by liquid ammonia-sodium and methyl iodide. The partially methylated T.K.P. from experiment II (1.05 g.) was suspended in liquid ammonia (20 ml.). To the suspension sodium (150 mg.) in small amounts was added with stirring followed by methyl iodide (0.4 ml.). After the reaction was over, ammonia was allowed to evaporate and the methylated polysaccharide extracted with chloroform. The chloroform extract was washed with water, dried over sodium sulfate and evaporated to yield a product (1.0 g.) having -OCH₃ 38.5%. The infrared spectrum showed appreciable hydroxyl absorption.
Experiment IV: Methylation by the Kuhn Method. The product from Experiment II (1.155 g.) was dissolved in dimethyl sulfoxide (DMSO) (15 ml.) and methylation carried out by adding a mixture of barium oxide-barium hydroxide (1:1, 5 g.) and methyl iodide (5 ml.) with constant stirring. The reaction was continued overnight after which ammonia was added to decompose excess of dimethyl sulfate. The reaction mixture was then poured into chloroform. The oily layer which separated was further extracted with chloroform. The chloroform extract, after washing with water till it attained neutrality, was dried over Na₂SO₄, filtered and evaporated. The yield of the methylated product was 0.905 g., -OCH₃: 39.5%.

Experiment V: Methylation by the DMSO Method. The combined partially methylated polysaccharide from Exp.I (5.2 g., -OCH₃: 39.5%) and Exp.II (2 g., -OCH₃: 37.2%) was dissolved in DMSO (100 ml.) and to the resulting solution sodium hydroxide pellets (30 g.) and dimethyl sulfate (15 ml.) were added during a period of 5 hours with constant vigorous stirring. The reaction mixture was stirred overnight and then heated to 95°C. for an hour to decompose excess of dimethyl sulfate. Water was added to dissolve the residual sodium hydroxide, cooled in ice-salt bath and partially neutralized with cold 10N sulfuric acid. Final neutralization was done by dilute acetic acid. The reaction mixture was filtered and the filtrate extracted with chloroform. The chloroform extract was washed with water, dried over Na₂SO₄, and evaporated to yield 5.35 g. of the methylated product.
having a methoxyl value of 43.0%. The infrared spectrum of the methylated product showed complete absence of hydroxyl adsorption.

Experiment VII: Direct methylation of T.K.P. by the D.M.S.O. method. 28

T.K.P. (1.2 g.) was dissolved in DMSO (100 ml.) and cooled to 20°C. Sodium hydroxide pellets (50 g.) and dimethyl sulfate (35 ml.) were added to small amounts during a period of six hours with constant vigorous stirring. For first three hours, the reaction was carried out at low temperature (bath temp. 20°C.) and in an atmosphere of nitrogen. After three hours, the cold bath was removed, and the reaction carried out at room temperature (50°C.). After overnight stirring, the reaction mixture was heated at 90-95°C. for an hour to decompose the excess of dimethyl sulfate. Water was added to dissolve the residual sodium hydroxide, and the resulting solution after cooling in an ice-salt bath was neutralized with cold 10N sulfuric acid. The reaction mixture was filtered and the filtrate extracted with chloroform. The chloroform extract was washed with water, dried over Na₂SO₄ and evaporated to yield 1.4 g. of methylated product having a methoxyl value of 41.5%. The infrared spectrum showed very little hydroxyl absorption.

FRACTIONATION OF METHYLATED T.K.P.

Methylated T.K.P. (5.2 g., Experiment V) was dissolved in dry acetone (100 ml.) and to the solution dry ether (50 ml.) was added. The inorganic impurities which precipitated out were centrifuged off. To the supernatant was added petroleum ether (50 ml., b.p. 40-60°C.) and
the material which separated was centrifuged off again to give fraction I. In the same way, by adding 20 ml., 40 ml., 60 ml. and 120 ml. of petroleum ether to the supernatants obtained after each addition, were obtained fractions II, III, IV, V & VI. After the sixth fraction was obtained, further addition of petroleum ether to the supernatant did not yield any precipitate. The supernatant was then evaporated to give fraction VII. The flow sheet of fractionation is given in Fig. 7. The specific rotations, methoxyl values and yields of different fractions are given in Table V.

TABLE V

FRACTIONATION OF METHYLATED TKP

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Yield (grams)</th>
<th>Methoxyl %</th>
<th>$\alpha$ in Chloroform</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.335</td>
<td>40.7</td>
<td>+22.8°</td>
</tr>
<tr>
<td>II</td>
<td>0.311</td>
<td>42.8</td>
<td>+29.3°</td>
</tr>
<tr>
<td>III</td>
<td>1.773</td>
<td>43.5</td>
<td>+29.5°</td>
</tr>
<tr>
<td>IV</td>
<td>0.919</td>
<td>43.5</td>
<td>+32.0°</td>
</tr>
<tr>
<td>V</td>
<td>0.593</td>
<td>43.6</td>
<td>+25.0°</td>
</tr>
<tr>
<td>VI</td>
<td>0.58</td>
<td>43.6</td>
<td>+25.4°</td>
</tr>
<tr>
<td>VII</td>
<td>0.914</td>
<td>55.7</td>
<td>+ 5.7°</td>
</tr>
</tbody>
</table>
FIG. 7

FRACTIONATION OF METHYLATED T.K.P.

Methylated TKP

\[ (5.2 \text{ g.}, \text{dissolved in 100 mL acetone}) \]

\[ +\text{ether (50 mL.)} \]

Residue
(Inorganic matter)

Supernatant

\[ +\text{Pet Ether (50 mL.)} \]

Supernatant

\[ +\text{Pet Ether (20 mL.)} \]

Residue
Fraction I
(0.3327 g.)

Supernatant

\[ +\text{Pet Ether (40 mL.)} \]

Residue
Fraction II
(0.3109 g.)

Supernatant

\[ +\text{Pet Ether (40 mL.)} \]

Residue
Fraction III
(1.7725 g.)

Supernatant

\[ +\text{Pet Ether (60 mL.)} \]

Residue
Fraction IV
(0.9193 g.)

Supernatant

\[ +\text{Pet Ether (120 mL.)} \]

Residue
Fraction V
(0.5950 g.)

Supernatant

Fraction VII
(0.9138 g.)
Fractions III, IV & VI, which were analytically similar, were combined and further investigations were carried out only on this material. Fraction V was reserved for determination of sugar ratios obtained on hydrolysis. The infrared spectra of all the fractions are given in Fig. 8.

**HYDROLYSIS OF METHYLATED T.K.P.**

Methylated T.K.P. (combined fractions III, IV and VI; 2.8 g.) was suspended in 72% sulfuric acid (13.2 ml.) and the suspension stored for half an hour at room temperature. It was diluted with water (105 ml.) and heated on a boiling water bath for 4 hours. The hydrolyzate, after neutralization with BaCO₃, was deionized by passing through Amberlite IR-120 and IR-45 resins and the resulting solution evaporated. The sirup, having μD +44.5° in methanol (c, 0.5), on chromatography in solvent C and spraying with reagent (a), showed the presence of a number of methylated sugars. (See Table VI).

**TABLE VI**

<table>
<thead>
<tr>
<th>Spot No. (in descending order)</th>
<th>Rf.</th>
<th>Solvent C</th>
<th>Probable Sugars</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.27</td>
<td></td>
<td>2,3-Di-0-methyl-D-glucose</td>
</tr>
<tr>
<td>2</td>
<td>0.54</td>
<td></td>
<td>2,3,6-Tri-0-methyl-D-glucose</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3,4-Di-0-methyl-D-xylose</td>
</tr>
<tr>
<td>3</td>
<td>0.67</td>
<td></td>
<td>2,3,4,6-Tetra-0-methyl-D-galactose</td>
</tr>
<tr>
<td>4</td>
<td>0.80</td>
<td></td>
<td>2,3,4-Tri-0-methyl-D-xylose</td>
</tr>
<tr>
<td>5</td>
<td>0.85</td>
<td></td>
<td>2,3,5-Tri-0-methyl-L-arabinose</td>
</tr>
</tbody>
</table>

42
DIFFERENT FRACTIONS OF METHYLATED T-K-P.
SEPARATION AND IDENTIFICATION OF METHYLATED SUGARS:

The hydrolyzate was resolved into its components by chromatography on a column of Whatman No.1 cellulose powder using solvent C as the developer. 10 Ml. fractions were collected. Each fraction was examined chromatographically and similar fractions were mixed and further purified either by paper chromatography or by paper electrophoresis and identified as described below:

(1) **2,3,5-Tri-O-methyl-\text{-}L-arabinose**

The substance isolated in the form of a sirup was dissolved in water, decolorized with charcoal and evaporated to yield a colorless sirup $\alpha_D -33.5^\circ$ in water ($c$, 0.4). On paper chromatogram, it moved as a single spot in solvents C and D. It had the same Rf value as an authentic sample of 2,3,5-tri-O-methyl-\text{-}L-arabinose. Demethylation with boron trichloride produced predominantly arabinose. The methylated sugar was refluxed with 2\% methanolic hydrochloride and the resulting methyl glycoside was examined by gas liquid chromatography using the Apiezon M column. The glycoside under examination had the same retention time as the authentic methyl 2,3,5-tri-O-methyl-\text{-}\alpha-\beta-\text{-}L-arabinoside.

(2) **2,3,4-Tri-O-methyl-D-xylose**

The sirupy product, having $\alpha_D +20^\circ$ in chloroform ($c$, 1), was chromatographically pure in solvents C and D and its Rf was identical with an authentic sample of 2,3,4-tri-O-methyl-D-xylose.
Demethylation with boron trichloride produced mainly xylose. The fraction crystallized on nucleation, and on recrystallization from ether-petroleum ether (b.p. 40-60°C) gave prisms, m.p. and mixed m.p. 91°, \( \left[ \alpha \right]_D^{20} +57.2° \rightarrow +23.4° \) in chloroform (c, 0.7). \([\text{Lit. Values}^{51} \text{ m.p. } 91-92°; \left[ \alpha \right]_D^{20} +55.0° \rightarrow +24.2° \text{ in } \text{CHCl}_3]\). The above sample (40 mg.) was refluxed with freshly distilled aniline (20.5 mg.) in super dry ethanol (5 ml.) for 5 hrs. The solution was evaporated and the resulting sirup, after dissolution in ether-petroleum ether, was nucleated with an authentic specimen to furnish needle shaped crystals, m.p. and mixed m.p. 100-101°. The infrared spectrum of this compound was identical with that of the authentic sample.

(3) \( \alpha,\beta,\gamma,\delta\)-Tetra-\(\alpha\)-methyl-\(\beta\)-galactose:

The sirupy product, having \( \left[ \alpha \right]_D^{20} +110.5° \) in water (c, 0.5), was chromatographically pure and had the same Rf value as that of an authentic sample of \( \alpha,\beta,\gamma,\delta\)-tetra-\(\alpha\)-methyl-\(\beta\)-galactose. Demethylation with boron trichloride produced mainly galactose. The methyl glycoside of the sugar derivative when examined by gas chromatography (Apiezon column) showed the same retention times as authentic samples of methyl-tetra-\(\alpha\)-methyl-\(\alpha\) and \(\beta\)-\(\delta\)-galactopyranosides.

The above sirup (25 mg.) was refluxed with freshly distilled aniline (11 mg.) in super dry ethanol (3 ml.) for 8 hours. The solution on slow evaporation in a desiccator, furnished needle shaped crystals. On recrystallization from ethyl acetate, the
crystals and m.p. and mixed m.p. 196°; $\Delta T_B = 67.5°$ in acetone (g, l.l) *Lit. value* 192°, $\Delta T_B - 77°$ in acetone. The infrared spectrum of this anilide derivative was identical with that of an authentic sample of this compound.

(4) Resolution of the mixture of 2,3,3-ri-O-methyl-D-glucose and 3,4-di-O-methyl-D-xylose.

The fraction, containing 2,3,6-tri-O-methyl-D-glucose, although moved as one spot on the paper chromatogram, the form of the spot was not regular and as such, heterogeneity could not be ruled out. In order to check this, the fraction was examined by paper electrophoresis which revealed that this fraction was a mixture of three components. (see Fig.9). Component A which corresponded to 2,3,6-tri-O-methyl-D-glucose, was present in maximum amounts; component B having $M_G$ value of 0.30, corresponded to 3,4-di-O-methyl-D-xylose and component (C) having $M_G$ value of 0.66 could not be identified as it was present only in trace amounts.

The mixture was resolved into its component by large scale paper electrophoresis. The different zones demarcated by guide strips, were cut out and eluted with water. The eluate was treated with Amberlite IR-120 resin and the effluent, after evaporation, repeatedly distilled with methanol to remove boric acid. Further deionization of the solution was carried out by passing through
RESOLUTION OF MIXTURE OF 3:4 DI-O-METHYL-D-XYLOSE AND 2:3:6 TRI-O-METHYL-D-GLUCOSE (FRACTION FROM CELLULOSE COLUMN) BY PAPER ELECTROPHORESIS 0.1M BORATE BUFFER - 600 VOLTS 3.5 HOURS

Amberlite IR-120 and IR-45 resins. The products were decolorised with charcoal and evaporated. The components A and B were obtained in a molar ratio of 2:1.

(A) Identification of 2,3,6-Tri-O-methyl-D-glucose:

The component A, having $\alpha_D^\infty +65^\circ$ in methanol (g.1), was chromatographically and electrophoretically pure and on demethylation with boron trichloride$^4$ gave mainly glucose.

The sirup (56 mg.) was dissolved in dry pyridine (5 ml.) and to the solution p-nitrobenzoyl chloride (450 mg.) was added. The mixture was heated to 80-90° for half an hour, cooled to room temperature and kept overnight. The excess of the reagent was destroyed by addition of a saturated solution of sodium bicarbonate and after adding water (10 ml.), the mixture was extracted with chloroform. The chloroform extract was washed with water, dried over $\text{Na}_2\text{SO}_4$, and evaporated. The sirup was dissolved in minimum amount of methanol, treated with charcoal and filtered hot. The filtrate upon cooling deposited crystals of 1,4-bis-O-p-nitrobenzoyl-2,3,6-tri-O-methyl-D-glucose, m.p. and mixed m.p. 190-192°, $\alpha_D^\infty -35.5^\circ$ in acetone (g, 0.8). $\angle$ Lit. value$^5$ $\alpha_D^\infty -55^\circ$ in acetone$^7$.

The infrared spectrum of this derivative was identical with that of an authentic sample of the 2,3,6-tri-O-methyl-D-glucose-1,4-bis-O-p-nitrobenzoate.
B) Identification of 3,4-Di-O-methyl-D-xylose:

The component B, having $\alpha_B^\circ = +19.5^\circ$ in methanol ($c_0.25$), was chromatographically and electrophoretically pure and on demethylation with boron trichloride produced only xylose.

The above substance (50 mg.) was oxidized with bromine (5 drops, in 5 ml. of water containing 50 mg. of BaCO$_3$) for 72 hours in the dark. Excess bromine was expelled by aeration and the mixture filtered. The filtrate was neutralized with silver carbonate, filtered and the filtrate, after deionizing with Amberlite IR-120 resin, was concentrated. The solution was extracted with chloroform and the chloroform extract was evaporated and lactonized by distillation, b.p. 140-160$^\circ$ (bath temp.) at 0.02-0.01 mm. of Hg. The distillate crystallized on standing and, after recrystallization from ether, yielded 3,4-di-O-methyl-D-xylopyranoside, m.p. and mixed m.p. 66-67$^\circ$.

D) 2,3-Di-O-methyl-D-glucose:

The sirup, $\alpha_B^\circ = +45.5^\circ$ in water ($c_1$), moved as a single spot both on paper chromatogram in solvents C & D and on ionophoretogram. It had the same mobility as an authentic sample of 2,3-di-O-methyl-D-glucose. Demethylation with boron trichloride produced only glucose.

The sirup was treated with 5% methanolic hydrochloride and the glycoside so formed, on crystallization from ether-pet.ether,
afforded plate-like crystals of methyl 2,3-di-O-methyl-\(\alpha\)-D-glucoside m.p. and mixed m.p. 85°C, \(\int \alpha_D^0 + 144^\circ\) in water (g.1).

\(\int \) Let. value\(^54\) m.p. 80-82°C, \(\int \alpha_D^0 + 142.6^\circ\) in water\(^7\).

Determination of Methylated Sugar Ratio:

Fraction V from the methylated polysaccharide was hydrolyzed by the method of Croon et al.,\(^29\) neutralized with barium carbonate, filtered and deionized with Amberlite IR-120 and IR-45 resins and concentrated. The mixture of sugars was resolved by paper chromatography in solvent C and the different zones containing methylated sugars were cut out and eluted with methanol. After filtration and evaporation, the individual components were extracted with water, filtered, and evaporated to constant weight.

The 2,3,6-tri-O-methyl-D-glucose had admixed with it 3,4-di-O-methyl-D-xylose (in a molar ratio of 2:1) as revealed by ionophoresis and described earlier. The molar ratios of different methylated sugars as computed from paper chromatography i.e. analysis in conjunction with ionophoresis, are given in Table VII.

**TABLE VII**

<table>
<thead>
<tr>
<th>Methylated Sugar</th>
<th>Molar Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 2,3,5-Tri-(\alpha)-methyl-(\alpha)-arabinose</td>
<td>0.85</td>
</tr>
<tr>
<td>2. 2,3,4-Tri-(\alpha)-methyl-(\alpha)-xylose</td>
<td>3.0</td>
</tr>
<tr>
<td>3. 2,3,4,6-Tetra-(\alpha)-methyl-(\alpha)-galactose</td>
<td>2.0</td>
</tr>
<tr>
<td>4. 3,4-Di-(\alpha)-methyl-(\alpha)-xylose</td>
<td>1.0</td>
</tr>
<tr>
<td>5. 2,3,6-Tri-(\alpha)-methyl-(\alpha)-glucose</td>
<td>2.0</td>
</tr>
<tr>
<td>6. 2,3-Di-(\alpha)-methyl-(\alpha)-glucose</td>
<td>6.0</td>
</tr>
</tbody>
</table>
DETERMINATION OF SUGAR RATIOS IN THE POLYSACCHARIDE
BY GAS CHROMATOGRAPHY

The hydrolyzate (50 mg.) of the methylated T.K.P. was methylated by Kuhn's method using DMF (5 ml.) BaO Ba(OH)$_2$·3H$_2$O (15:1; 500 mg.) and methyl iodide (5 ml.) for 16 hours. The reaction mixture was poured in chloroform and the filtrate evaporated. The mixture when resolved by gas-liquid chromatography using butane diol succinate column, showed peaks corresponding to different methylated sugars (see Fig. 16). The analysis revealed the presence of traces of arabinopyranose residues also. The ratio of different sugars as calculated on the basis of peak areas is given in Table VIII.

TABLE VIII

GAS CHROMATOGRAPHIC ANALYSIS OF METHYLATED T.K.P. AFTER HYDROLYSIS AND REMETHYLATION.

<table>
<thead>
<tr>
<th>Fully Methylated Methyl Glycosides of</th>
<th>Peak No.</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-arabinofuranose</td>
<td>I</td>
<td>1.0</td>
</tr>
<tr>
<td>L-arabinopyranose</td>
<td>IV</td>
<td></td>
</tr>
<tr>
<td>β-D-xylose</td>
<td>II</td>
<td>4.2</td>
</tr>
<tr>
<td>α-D-xylose</td>
<td>III</td>
<td></td>
</tr>
<tr>
<td>β-D-glucose</td>
<td>V</td>
<td>8.5</td>
</tr>
<tr>
<td>α-D-glucose</td>
<td>VI</td>
<td></td>
</tr>
<tr>
<td>β-D-galactose</td>
<td>VII</td>
<td>2.2</td>
</tr>
</tbody>
</table>
FULLY METHYLATED METHYL GLYCOSIDES OF
(1)α,β-L-ARABINOFURANOSE (2)β-D-XYLOSE (3)α-D-XYLOSE
(4) L-ARABINOPYRANOSE (5)β-D-GLUCOSE (6)α-D-GLUCOSE
(7) α,β-D-GALACTOSE

FIG. 10
A 1% solution of T.K.P. in 0.01N sulfuric acid was heated on a water bath maintained at 80°C. After every two hours ca. 25 ml. samples were withdrawn, neutralized with BaCO₃ and filtered. The filtrates were concentrated and examined by paper chromatography in solvents A and B using spray reagents (a) and (b). In all 25 samples were taken out and examined chromatographically. While the first sample showed the presence of arabinose only, the second and third samples were composed of arabinose and galactose. Samples 4-25 showed the presence of arabinose, galactose and xylose. No glucose was detected in any of the hydrolysates even on heavy spotting of the samples.
ACETOLYSIS

Four one gm. samples of T.K.P. were shaken with acetic anhydride (5 ml.), acetic acid (5.6 ml.) and concentrated sulfuric acid (0.28 ml.) for four days. The samples were then mixed and centrifuged to remove the insoluble matter. The supernatant was poured into crushed ice and the mixture allowed to stand for one hour with occasional stirring. It was then extracted with chloroform, the chloroform extract washed successively with sodium bicarbonate solution and with water, and the extract, after drying over Na₂SO₄, was concentrated to a sirup (0.90 g.). Deacetylation of the product with sodium methoxide gave a mixture of monosaccharides and oligosaccharides, which were examined chromatographically in solvent A. The results are given in Table IX.

TABLE IX

CHROMATOGRAPHIC ANALYSIS OF THE COMPONENTS PRODUCED ON ACETOLYSIS OF T.K.P.

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>R_cellobiose</th>
<th>R_glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.76</td>
<td>0.57</td>
</tr>
<tr>
<td>2</td>
<td>1.00</td>
<td>0.48</td>
</tr>
<tr>
<td>3</td>
<td>1.45</td>
<td>0.68</td>
</tr>
</tbody>
</table>

4, 5, 6 and 7 were galactose, glucose, arabinose and xylose respectively.
While the intensity of spot no. 1 indicated that this oligosaccharide was present in only minor quantity, oligosaccharides A and B were present in comparatively larger amounts. The monosaccharides constituted the major part of the hydrolyzate.

Separation of the Oligosaccharides A and B on Carbon Column.

The sugar mixture was resolved on a charcoal celite (50:50) column. The column was eluted successively with 2.5% aqueous ethanol and 5% aqueous ethanol. 100-150 ml. fractions were collected and after evaporation were examined by paper chromatography. The results are given in Table I.

TABLE I
RESOLUTION OF THE SUGAR MIXTURE OBTAINED BY ACETOLYSIS OF T.K.P. BY CARBON COLUMN CHROMATOGRAPHY.

<table>
<thead>
<tr>
<th>Elution by 2.5% Aqueous Ethanol</th>
<th>Elution by 5% Aqueous Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fractions</td>
<td>Sugar present</td>
</tr>
<tr>
<td>----------</td>
<td>---------------</td>
</tr>
<tr>
<td>1-19</td>
<td>Monosaccharides</td>
</tr>
<tr>
<td>20-24</td>
<td>Galactose Oligosaccharide B</td>
</tr>
</tbody>
</table>

Further Separation of Oligosaccharides by Paper Chromatography

The mixture of oligosaccharides was further resolved on Whatman No. 1 filter paper in solvent A. The different oligosaccharide
zones were cut out, eluted with water and evaporated. The similar fractions were pooled together to give oligosaccharide A (58 mg.) and oligosaccharide B (42 mg.).

Characterization of 4-O-β-D-glucopyranosyl-β-D-glucose (CELLOBIOSE)

The syrup having \( \left[ \alpha \right]_D^{54.0^\circ} \) in water \((c, 0.5)\), was chromatographically indistinguishable with cellobiose in solvents A and B.

Acid hydrolysis with \( \mathrm{N} \) sulfuric acid produced only glucose.

The syrup \((50 \text{ mg.})\) was acetylated with acetic anhydride \((5 \text{ ml.})\) and anhydrous sodium acetate \((500 \text{ mg.})\) by heating on a boiling water bath for 8 hours. The reaction mixture was cooled and poured into crushed ice. After standing for 2 hours, the reaction mixture was extracted with chloroform and the extract, after washing with water and drying over \( \mathrm{Na}_2\mathrm{SO}_4 \), was evaporated. The crystalline mass thus obtained was recrystallized from hot ethanol to furnish crystals of octa-O-acetyl-β-cellobiose \(56^\circ\) m.p. and mixed m.p. 188-189\(^\circ\).

Identification of 2-O-β-D-galactopyranosyl-β-xylopyranose

The syrup having \( \left[ \alpha \right]_D^{+52.0^\circ} \) in water \((c, 0.5)\) upon hydrolysis with \( \mathrm{N} \) sulfuric acid produced galactose and xylose in equimolar proportions as determined by quantitative paper chromatographic analysis.

Reduction of the oligosaccharide with sodium borohydride,
followed by acid hydrolysis with H sulfuric acid produced only galactose as shown by paper chromatography.

A spot of the oligosaccharide on paper, when sprayed with triphenyl tetrazolium chloride gave a negative test, while with reagent (a) it produced the characteristic pink colour of pentoses.

The sirup (28 mg.) was methylated by Kuhn's method. It was dissolved in DMF (2 ml.) and methylated by adding barium oxide-barium hydroxide (15:1; 300 mg.) and methyl iodide (1 ml.), with constant stirring. After overnight stirring, the reaction mixture was poured into chloroform and the inorganic salts which separated were filtered. The filtrate, after washing with sodium thiosulfate solution (to remove iodine) and with water, was dried over sodium sulfate. The sirup obtained on evaporation of the chloroform solution, was further methylated by the Purdie reagents. After two Purdie methylations, complete methylation was achieved as shown by lack of a hydroxyl peak in the infrared spectrum of the product.

Hydrolysis of the methylated disaccharide by the method of Croon et al., yielded 2,3,4,6-tetra-O-methyl-D-galactose and 3,4-di-O-methyl-D-xylose as identified by paper chromatography in two solvents C and D and by ionophoresis.
ENZYMIC HYDROLYSIS

A 1% solution of the polysaccharide in water along with hemicellulose* (10% on the weight of the polysaccharide) was taken in a cellophane tube. After covering the solution with toluene, the contents were dialyzed against distilled water contained in a cylindrical glass vessel. The apparatus (Fig. 11) was put in a thermostat maintained at 40°C. The water outside the dialysis tube was changed periodically to enable fractionation of digested material. The contents of the cellophane tube were shaken occasionally to resuspend the precipitated material. For the first nine fractions, the water outside the cellophane tube was changed every three days and for the rest of the fractions after every six days. The dialyzates were concentrated and examined chromatographically (see Figs. 12a and 12b). The results of the enzymic hydrolysis are given in Table XI.

TABLE XI

CHROMATOGRAPHIC ANALYSIS OF FRACTIONS FROM ENZYMIC HYDROLYSIS OF T.K.P.

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Days</th>
<th>Sugars present</th>
<th>( R_{celllobiose} (R_c) ) in solvent A</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2</td>
<td>0-6</td>
<td>Oligosaccharide C</td>
<td>0.80</td>
</tr>
<tr>
<td>3</td>
<td>6-9</td>
<td>Oligosaccharide C</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oligosaccharide D</td>
<td>1.22</td>
</tr>
<tr>
<td>4</td>
<td>9-12</td>
<td>Oligosaccharides C and D glucose and arabinose</td>
<td></td>
</tr>
<tr>
<td>5,6,7</td>
<td>12-21</td>
<td>Oligosaccharide D glucose, arabinose and xylose</td>
<td></td>
</tr>
<tr>
<td>8-13</td>
<td>21-51</td>
<td>Oligosaccharide D (Traces of monosaccharides)</td>
<td></td>
</tr>
</tbody>
</table>

*An enzyme preparation of the Nutritional Biochemicals Corporation.
APPARATUS FOR ENZYMIC HYDROLYSIS

FIG. 11
ENZYMIC HYDROLYSIS OF TAMARIND POLYSACCHARIDE WITH HEMICELLULASE AT 40°C
SAMPLES TAKEN OUT AFTER EVERY 3 DAYS
CHROMATOGRAPHY IN BUTANOL: PYRIDINE: WATER (6:4:3), 48 HRS.
STANDARD SUGARS - CELLOMINOSE, GLUCOSE, ARABINOSE, XYLOSE
IN DESCENDING ORDER
ENZYMIC HYDROLYSIS OF TAMARIND POLYSACCHARIDE WITH HEMICELLULASE AT 40°C
SAMPLES 8 & 9 TAKEN OUT AFTER EVERY 3 DAYS
SAMPLES 10 TO 14 TAKEN OUT AFTER EVERY 6 DAYS
CHROMATOGRAPHY IN BUTANOL: PYRIDINE: WATER (6:4:3) 48 HRS.
STANDARD SUGARS-CELLOBIOSE, GALACTOSE, GLUCOSE,
ARABINOSE & XYLOSE IN DESCENDING ORDER

FIG. 12b
Fractions of similar composition were pooled together and further purified on Whatman No. 5 filter paper using solvent A. In this manner oligosaccharides C and D were obtained in a pure state, the yields being 14% and 40% respectively on the basis of the weight of the polysaccharide.

Identification of 4-O-β-D-Galactopyranosyl-D-glucose (LACTOSE)

The oligosaccharide C, having a Re of 0.80 in solvent A, was chromatographically and electrophoretically indistinguishable from authentic lactose.

Acid hydrolysis of the oligosaccharide with N sulfuric acid produced glucose and galactose in equimolar proportions as determined by the phenol-sulfuric acid method.

The degree of polymerisation as determined by Timell's method was 1.78. Lactose by the same method gave a value of 1.8.

When the disaccharide was reduced with sodium borohydride and hydrolyzed with N sulfuric acid, only galactose was detected in the hydrolyzate.

A spot on paper, when sprayed with triphenyl tetrazolium chloride reagent, produced a pink colour and with aniline-diphenylamine phosphate reagent, produced a greenish blue colour.

The disaccharide crystallized from aqueous ethanol on keeping
in the cold for 3 weeks. The crystals had m.p. 200-261° and when mixed with an authentic sample of lactose, did not depress the melting point. The sugar had \( \alpha_7^\infty +80.5^\circ \rightarrow +49.5^\circ \) in water (c,1). Lit. value\(^57\) m.p. 201°C, \( \alpha_7^\infty +80.8^\circ \rightarrow +51.23^\circ \).

**Analysis** calcd. for C\(_{12}\)H\(_{22}\)O\(_{11}\).H\(_2\)O: C, 40.0; H, 6.66.

Found: C, 40.5; H, 7.0.

The infrared spectrum of the disaccharide was identical with that of an authentic example of lactose. (Fig.15).

The disaccharide (20 mg.) in water (0.3 ml.) was treated with phenylhydrazine (4 molar equivalents) and glacial acetic acid (4 molar equivalents) in a boiling water bath for 2 hours. On cooling, bright yellow crystals separated, which on filtration and recrystallization from aqueous ethanol, gave yellow crystals m.p. (decom.) 208-211° which did not depress when mixed with an authentic sample of lactosazone.

**Methylation of Lactose (Disaccharide C)**

Disaccharide C (80 mg.) was dissolved in dimethyl sulfoxide (2 ml.) and to the solution BaO (200 mg.) and methylidide (1 ml.) were added. The reaction mixture, after overnight stirring, was poured into chloroform and the precipitated salts filtered off. The filtrate was washed with dil. sodium thiosulfate solution and then with water. The chloroform solution, after drying (Na\(_2\)SO\(_4\)) was evaporated to dryness. The residue was then treated three times
INFRARED SPECTRA OF LACTOSE OBTAINED BY ENZYMIC HYDROLYSIS OF T.K.P.

AUTHENTIC SAMPLE

INFRARED SPECTRA OF LACTOSE OBTAINED BY ENZYMIC HYDROLYSIS OF T.K.P.

AUTHENTIC SAMPLE

FIG. 13
with Purdie reagents to complete the methylation. The methylated product (81 mg.) did not show any hydroxyl absorption in the infrared region.

**Hydrolysis of the Methylated Disaccharide**

The methylated disaccharide was hydrolyzed by the method of Croon et al.²⁹ Paper chromatographic examination of the hydrolysate showed the presence of 2,3,4,6-tetra-α-methyl-D-galactose and 2,3,6-tri-α-methyl-D-glucose. The methylated fragments, which were present in equimolecular proportions were isolated by isolative paper chromatography. 2,3,4,6-tetra-α-methyl-D-galactose was characterized by preparing its anilide derivative, m.p. and mixed m.p. 196°C, $\left[\alpha\right]_D^{o} -87.0^\circ$ in acetone.

The 2,3,6-tri-α-methyl-D-glucose fraction was treated with p-nitrobenzoyl chloride in pyridine to give 1,4 bis p-nitrobenzoyl 2,3,6-tri-α-methyl-D-glucose, m.p. and mixed m.p. 190°C, $\left[\alpha\right]_D^{o} -35^\circ$ in acetone.

**Identification of 6-α-D-Xylopyranosyl-D-Glucopyranose**

The sirup, having $\left[\alpha\right]_D^{o} +122^\circ$ in water (c,1), moved as one spot on paper chromatogram in solvents A and B (Rc in solvent A,1.22) and on phoretogram in borate buffer.

Acid hydrolysis of the oligosaccharide with HN sulfuric acid produced glucose and xylose in equimolar proportions as estimated by the phenol-sulfuric acid method.²²
The oligosaccharide had a D.P. of 2 as found by the method of Timell\textsuperscript{26} for D.P. determinations.

The disaccharide (60 mg.) was dissolved in DMF (2 ml.) and methylated by Kuhn's method\textsuperscript{23} using barium oxide-barium hydroxide mixture (15:1; 500 mg.) and methyl iodide (2 ml.) with constant stirring. After overnight stirring the reaction mixture was poured into chloroform and the precipitated material filtered. The filtrate, after washing successively with thiosulfate solution to remove iodine, and with water was dried (Na\textsubscript{2}SO\textsubscript{4}) and then evaporated. The methylated product was once again methylated by Purdie reagents to give a fully methylated disaccharide as evidenced by the absence of hydroxyl absorption in the infrared region. The methylated disaccharide was hydrolyzed by Croon's method\textsuperscript{29} in the usual manner. Chromatographic examination of the hydrolyzate showed the presence of 2,3,4-tri-O-methyl-D-xylose and 2,3,4-tri-O-methyl-D-glucose. The mixture of methylated sugars was resolved on Whatman No.1 filter paper in solvent C to get 2,3,4-tri-O-methyl-D-glucose (28 mg.) and 2,3,4-tri-O-methyl-D-xylose (25 mg.).

2,3,4-tri-O-methyl-D-glucose was refluxed with ethanolic aniline for 3 hours and on slow evaporation of the solution in a desiccator provided crystals which on recrystallization from ethyl ether furnished crystals of N-phenyl 2,3,4-tri-O-methyl-D-glucosylamine m.p. 144-145\degree; (reported m.p. 145-146\degree).\textsuperscript{58}
2,3,4-tri-\(\alpha\)-methyl-\(\beta\)-xylose was also treated with ethanolic aniline, as described above, to afford the characteristic crystalline amilide, m.p. and mixed m.p. 100-101\(\degree\).
PERIODATE OXIDATION

T.K.P. (1 g.) was dissolved in water (200 ml.) and the solution cooled to 0°C. A cold solution of sodium metaperiodate (0.124 M, 200 ml.) was added to the polysaccharide solution. After making up the volume to 500 ml., the flask was stored in a refrigerator at 10°C. A blank was also set up in the similar way. Aliquots (10 ml.) were pipetted out at different time intervals to determine the amounts of periodate consumed and formic acid liberated.

1. Determination of Periodate Consumption by the Method of Fleury and Lange.59

The aliquot was taken in a conical flask containing 0.1N sodium arsenite solution (10 ml.), saturated sodium bicarbonate solution (10 ml.) and potassium iodide (1 g.). The flask was kept in the dark for 15 minutes and then excess of arsenite titrated with standard iodine solution using starch as indicator.

2. Determination of Formic Acid60

The aliquot was taken in a conical flask containing ethylene glycol (2 ml.) and allowed to stand for 10 minutes in the dark. Potassium iodide (1 g.) and .05N sodium thiosulfate solution (5 ml.) were added and the resulting solution titrated against standard iodine solution using starch as indicator.

The periodate consumed and formic acid liberated per mole of
The anhydro sugar with time are given in Table XII.

<table>
<thead>
<tr>
<th>Time in hrs.</th>
<th>12</th>
<th>25</th>
<th>75</th>
<th>96</th>
<th>121</th>
<th>145</th>
<th>169</th>
</tr>
</thead>
<tbody>
<tr>
<td>I(_4) consumed mole anhydro sugar</td>
<td>1.05</td>
<td>1.22</td>
<td>1.26</td>
<td>1.30</td>
<td>1.33</td>
<td>1.35</td>
<td>1.35</td>
</tr>
<tr>
<td>HCOOH liberated/ mole anhydro sugar</td>
<td>0.30</td>
<td>0.312</td>
<td>0.32</td>
<td>0.328</td>
<td>0.337</td>
<td>0.34</td>
<td>0.34</td>
</tr>
</tbody>
</table>

After seven days, the reaction mixture was treated with Ba\(\text{CO}_3\), the precipitates of barium iodate and periodate were filtered and the filtrate deionized with Amberlite IR-120 and IR-45 resins. The resulting solution was reduced with sodium borohydride and after neutralization with acetic acid and deionizing with Amberlite IR-120 resin, the solution was evaporated to dryness. Boric acid was removed by repeated distillation with methanol. The residue was hydrolyzed with N sulfuric acid. The hydrolysate was neutralized with Ba\(\text{CO}_3\), filtered and deionized with Amberlite IR-120 and IR-45 resins. The solution was concentrated and the sirup, when examined paper chromatographically in solvent A and sprayed with reagents B and C, revealed the presence of glyceritol, erythritol and glyceraldehyde only.
**Determination of glycerol:erythritol ratio**

(A) **Preparation of standard curves for glycerol and erythritol according to the method of Lambert and Heish**\(^{61}\)

A known amount of standard solution of the polyalcohol was taken in a volumetric flask (100 ml.) and the volume made up to 20 ml. with water. To the solution, after acidification with 10M sulfuric acid (1 ml.), were added with mixing 0.1M sodium metaperiodate solution (5 ml.) and exactly after 5 minutes, 1M sodium arsenite solution (5 ml.). After 10 minutes, the volume was adjusted to 100 ml. with water and mixed thoroughly. 1 ml. aliquot was pipetted out in test tubes in triplicate and chromotropic acid reagent (10 ml.) was added with mixing. The tubes were then heated over a boiling water bath for half an hour, in the dark. The tubes were then cooled in water and the absorbance read at 570 nm. A blank was also prepared in a similar manner and was used to adjust the colorimeter to zero. The absorbance values for glycerol and erythritol for different amounts are given in Tables XIII and XIV. (see Fig.14).

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Amount of Glycerol (Micrograms)</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.208</td>
<td>0.0962</td>
</tr>
<tr>
<td>2</td>
<td>4.416</td>
<td>0.1869</td>
</tr>
<tr>
<td>3</td>
<td>6.624</td>
<td>0.2758</td>
</tr>
<tr>
<td>4</td>
<td>8.832</td>
<td>0.3717</td>
</tr>
<tr>
<td>5</td>
<td>11.040</td>
<td>0.4559</td>
</tr>
</tbody>
</table>
STANDARD CURVES FOR GLYCEROL AND ERYTHRITOL

FIG. 14
**TABLE XIV**

**STANDARD CURVE FOR ERYTHRITOL**

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Amount of Erythritol (Micrograms)</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.0</td>
<td>0.0825</td>
</tr>
<tr>
<td>2</td>
<td>4.2</td>
<td>0.1268</td>
</tr>
<tr>
<td>3</td>
<td>6.0</td>
<td>0.1772</td>
</tr>
<tr>
<td>4</td>
<td>7.2</td>
<td>0.2256</td>
</tr>
<tr>
<td>5</td>
<td>9.0</td>
<td>0.2840</td>
</tr>
<tr>
<td>6</td>
<td>12.0</td>
<td>0.3615</td>
</tr>
</tbody>
</table>

(B) **Determination of glycerol-erythritol-glyceraldehyde ratio in the hydrolysate of reduced oxopolysaccharide**

As glyceraldehyde moved very close to glycerol, it was necessary to remove it from the mixture. For this purpose, the hydrolysate was divided into two portions A and B.

(1) **Portion A** was oxidized with bromine water in the presence of BaCO₃ for 48 hours. Bromine was removed by aeration and the mixture was filtered. The filtrate was passed through Amberlite IR-120 and IR-45 resins to remove inorganic and organic acids such as glycolic acid and glyceric acid. The effluent was concentrated and the resulting sirup resolved on Whatman No.1 filter paper using solvent A. The different zones for glycerol and erythritol were cut out, eluted with water and the ratio of glycerol to erythritol determined by the chromotropic acid procedure.
(2) Portion B was reduced with sodium borohydride, neutralized with acetic acid, deionized with Amberlite IR-120 resin and evaporated to dryness. Boric acid was removed by repeated distillations with methanol. The residue was dissolved in water and the solution deionized with Amberlite IR-120 and IR-45 resins. The solution was concentrated and the ratio of glycerol to erythritol was determined as described earlier. The results are given in Table XV.

**TABLE XV**

<table>
<thead>
<tr>
<th>Hydrolyzate</th>
<th>Molar Ratio</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Erythritol : Glycerol</td>
<td></td>
</tr>
<tr>
<td>Portion A (after oxidation)</td>
<td>1 : 0.317</td>
<td></td>
</tr>
<tr>
<td>Portion B (after reduction)</td>
<td>1 : 0.43</td>
<td></td>
</tr>
</tbody>
</table>

Glycerol arising from glyceraldehyde = 0.43-0.317 = 0.17

Proportion of glyceraldehyde = \( \frac{0.17 \times 90}{92} \) = 0.110

Thus erythritol : glycerol : glyceraldehyde

1 : 0.317 : 0.110

As we see that only the amount of glycerol is changed, due to the reduction of glyceraldehyde to glycerol, without affecting the
amount of erythritol, if we take that all the eight glucose units will give rise to erythritol after Smith degradation\textsuperscript{30} of the polysaccharide, eight erythritol units will be liberated. So the ratio of erythritol to glycerol to glyceraldehyde (1:0.317:0.11) given in Table XV can be changed to 8:2.55:0.88.
PARTIAL ACID HYDROLYSIS OF T.E.P.

To a solution of T.E.P. (20 g.) in water (2 l.) 2N sulfuric acid (500 ml.) was added with stirring and the solution was heated on a boiling water bath for 5 hours. The hydrolysate was neutralized (BaCO₃) and after concentration to a small volume was poured into alcohol. The precipitated polysaccharide was recovered by centrifugation followed by drying the usual solvent exchange technique yield 2.5 g. [α]D⁺35.5° in 0.5N NaOH (c,1) 0.5 NaOH). Hydrolysis of the partially degraded polysaccharide with H₂SO₄ for 10 hrs. in a sealed tube produced glucose and xylose in a molar ratio of 15:1; galactose and arabinose were not detected in the hydrolysate.

Fractionation of Degraded T.E.P.

The degraded polysaccharide (1 g.), obtained as above, was dissolved in water (75 ml.) and fractionated by the alcohol precipitation method. Five fractions I-V were obtained. The fractions were hydrolyzed with acid and the hydrolysates, after neutralization, examined chromatographically. The analytical data are presented in Table XVI.
TABLE XVI
FRACTIONATION OF DEGRADED T.K.P.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Alcohol (ml)</th>
<th>$\langle \alpha \rangle_D$ in water</th>
<th>Sugars produced on hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>150</td>
<td>+ 40.0</td>
<td>Glucose + Xylose</td>
</tr>
<tr>
<td>II</td>
<td>50</td>
<td>+ 28.3</td>
<td>-do-</td>
</tr>
<tr>
<td>III</td>
<td>50</td>
<td>+ 28.3</td>
<td>-do-</td>
</tr>
<tr>
<td>IV</td>
<td>150</td>
<td>+ 24.0</td>
<td>-do-</td>
</tr>
<tr>
<td>V</td>
<td>Residue</td>
<td>+ 24.0</td>
<td>-do-</td>
</tr>
</tbody>
</table>

Methylation of Degraded T.K.P.

Degraded T.K.P. (1 g.) was dissolved in DMSO and methylated by BaO (6 g.) and methyl iodide (10 ml.). The partially methylated product was given three Purdie methylations after which the permethylation was complete as indicated by absence of hydroxyl adsorption in the infrared. (yield 950 mg.).

Fractionation of Methylated Polysaccharide

The methylated product from the previous experiment was dissolved in acetone (25 ml.) and fractional precipitation carried out by adding increasing amounts of petroleum ether (40-60°C). Four fractions (I-IV) were obtained. (see Table XVII).
TABLE XVII

FRACTIONATION OF THE METHYLATED DEGRADED T.K.P.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Pet. Ether (ml.)</th>
<th>Yield (gms.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>20</td>
<td>0.116</td>
</tr>
<tr>
<td>II</td>
<td>10</td>
<td>0.20</td>
</tr>
<tr>
<td>III</td>
<td>20</td>
<td>0.338</td>
</tr>
<tr>
<td>IV</td>
<td>Residue</td>
<td>0.140</td>
</tr>
</tbody>
</table>

The fractions were hydrolysed by the method of Croon et al.\textsuperscript{29} and the hydrolysates examined chromatographically. All the fractions produced on hydrolysis 2,3,4,6-tetra-\(\alpha\)-methyl-D-glucose, 2,3,4-tri-\(\alpha\)-methyl-D-\(\alpha\)-glucose, 2,3,6-tri-\(\alpha\)-methyl-D-glucose and 2,3,4-di-\(\alpha\)-methyl-D-glucose. No 2,3,4-tri-\(\alpha\)-methyl-D-glucose could be detected chromatographically even on heavy spotting. Visual examination of the chromatogram revealed that 2,3,6-tri-\(\alpha\)-methyl-D-glucose was the main component along with a little 2,3,4-tri-\(\alpha\)-methyl-D-\(\alpha\)-glucose and 2,3,4-tri-\(\alpha\)-methyl-D-\(\alpha\)-xylose. 2,3,4,6-tetra-\(\alpha\)-methyl-D-glucose was present in relatively very small amounts as it could be detected on the paper only on heavy spotting.
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