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# An Aldotetraouronic Acid from the Hydrolyzate of a Paper Birch 4-*O*-Methylglucuronoxylan

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June, 1964

# AN ALDOTETRAOURONIC ACID FROM THE HYDROLYZATE OF A PAPER BIRCH 4-O-METHYLGLUCURONOXYLAN

A thesis submitted by

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## SUMMARY

A 4-O-methylglucuronoxylan was isolated directly from paper birch. The wood meal was pre-extracted with 0.1N sodium hydroxide and then extracted with 10% potassium hydroxide. The hemicellulose was precipitated from the neutralized extract with ethanol and isolated by filtration. Graded acid hydrolysis of the 4-O-methylglucuronoxylan yielded the usual spectrum of oligosaccharides. The tetrasaccharide acid fraction was isolated and purified by paper chromatography and paper electrophoresis; yield (based on the hemicellulose): 0.86%; $[\alpha]_D^{25}$ = 24°; equivalent weight 604.

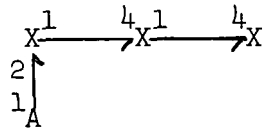
The structure of the aldotetraouronic acid was examined through a methylation study. A 150-mg. sample of the acid was converted to the nonreducing analog with alkaline sodium borohydride. The product was exhaustively methylated with sodium hydroxide and dimethyl sulfate, followed by silver oxide and methyl iodide in dimethylformamide. Reduction with lithium aluminum hydride yielded the fully methylated neutral tetrasaccharide. The component monosaccharides were obtained by cleavage of the glycosidic linkages by methanolysis.

The methanolysis mixture was analyzed and fractionated by gas chromatography. Approximately equimolar quantities of 2,3,4-tri-O-methyl-xylose, 3-O-methyl-xylose, 2,3,4-tri-O-methyl-glucose, and 1,2,3,5-tetra-O-methyl-xylitol were obtained. Small quantities of 2,3- and 3,4-di-O-methyl-xylose were also present. The four principal methylated components, representing 94 mole per cent of the material isolated, were identified by paper chromatography, melting points, and IR spectra. The two dimethyl xyloses, representing six mole per cent of the material isolated, were identified only by gas and paper chromatography.

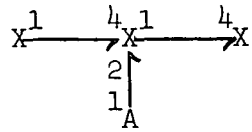
The methylation data indicated that the purified aldotetraouronic acid consisted of two components, of which Isomer A represented six per cent and Isomer

B represented 94%. The third possible tetrasaccharide acid, Isomer C, was not found.

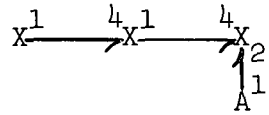
Isomer A



Isomer B



Isomer C



X = xylopyranose unit
A = 4-O-methylglucuronic acid unit

Mild acid hydrolysis of the aldotetraouronic acid yielded a 1:1 ratio of xylose and the aldotriouronic acid. Similar treatment of the nonreducing analog of the aldotetraouronic acid yielded xylose and the nonreducing analog of the aldotriouronic acid. These data also support the structure of Isomer B as the principal component of the purified aldotetraouronic acid.

The isolation of Isomer B represented the first direct confirmation of the postulated structure of the 4-O-methylglucuronoxylan. Oligouronide fragments previously isolated from the acid hydrolyzate of this hemicellulose contained the acidic substituent on the nonreducing end xylose unit, as in Isomer A. The stability of Isomer B, over the two possible alternate tetrasaccharide isomers, was attributed to steric factors.

# INTRODUCTION

The hydrolysis of polysaccharides by dilute mineral acid is of considerable practical as well as theoretical interest. Since these naturally occurring polymers are composed of sugar units connected by acid-labile glycosidic linkages, acidic pulping procedures may lead to extensive degradation. The associated removal of material may represent an appreciable loss in pulp yields. One of the most important of these polysaccharides is the hemicellulose, 4-O-methylglucuronoxylan. This work utilized a typical example of this polymer, that obtained from paper birch, in an investigation of the acid-catalyzed hydrolysis mechanism.

# ACID HYDROLYSIS OF GLYCOSIDES

Shafizadeh (1) and Capon and Overend (2) have discussed in detail the various factors affecting the acid-catalyzed hydrolysis of the glycosidic linkage. The appropriate essentials of these reviews, together with more recent information, are presented below.

## MECHANISM

Two general mechanisms, A and B below, may be postulated for the acid-catalyzed hydrolysis of glycosides (3, 4). The common first step in these two mechanisms is the establishment of the equilibrium between the glycoside, G, and its conjugate acid, $\underline{GH}^+$:

$$\underline{G} + H_3O^+ \longrightarrow \underline{GH}^+ + H_2O \qquad \text{(fast)}$$

The conjugate acid is then postulated to proceed through a reaction sequence in which the rate-controlling step is either (1) unimolecular heterolysis, as in A,

or (2) bimolecular substitution, as in B:

A $\underline{GH}^+ \longrightarrow \underline{G}^+$ (slow) (1)

$\underline{G}^+ + H_2O \longrightarrow$ products (fast)

($\underline{G}^+$ = carbonium ion intermediate)

B $\underline{GH}^+ + H_2O \longrightarrow$ products (slow) (2)

The molecularity of the hydrolysis mechanism, i.e., the validity of either A or B, may be determined by the criterion based on the Hammett acidity function, $\underline{H}_o$. In A the logarithm of the rate constant should be proportional to $\underline{H}_o$. In B the logarithm of the rate constant should be proportional to the pH. This criterion has been used by Bunton, et al., to establish Mechanism A for the acid hydrolysis of methyl and phenyl glucosides.

The site of scission, in the hydrolysis of methyl and phenyl glucosides, has been demonstrated to be between $C_1$ of the hexose and the glucosyl oxygen (4). Hydrolysis of these glycosides in a solution of water enriched with $H_2O^{18}$ yielded $O^{18}$-free methanol and phenol. The oxygen associated with the aglycon was, therefore, derived from the glucoside, and fission must have occurred as shown in Fig. 1. This mechanism has been established for methyl α- and β-D-glucopyranosides (4), phenyl α- and β-D-glucopyranosides (4), maltose (5), and methyl 2-deoxy-α-D-glucopyranoside (5).

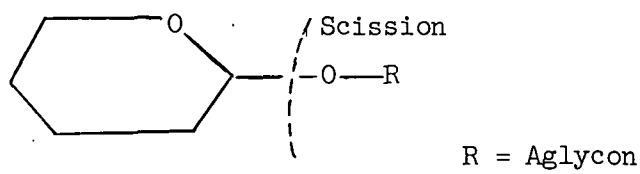



Figure 1. Site of Aglycon Fission

The conclusions of Bunton, et al. (4), were consistent with the two reaction mechanisms illustrated in Fig. 2. Both of these reaction sequences represent elaborations of the general Mechanism A, previously discussed. As before, the initial step involves rapid protonation of the original glycoside to yield the two possible conjugate acids. The acyclic mechanism then proceeds through a series of reactions in which opening of the pyranose ring is postulated to be rate controlling. The cyclic mechanism proceeds through a reaction series in which splitting off of the aglycon determines the rate. Both of these mechanisms converge to yield a second equilibrium between the parent pyranose and the two analogous conjugate acids.

Banks, et al. (6) have suggested that the cyclic mechanism of Fig. 2 may govern hydrolysis of methyl glucopyranosides. Substitution of $O^{18}$ for $O^{16}$ at the glucosyl oxygen produced a significant decrease in the reaction rate (oxygen isotope effect) during partial acid hydrolysis of methyl $\alpha$-D-glucopyranoside. This decrease was too large to be compatible with the acyclic mechanism but was consistent with the isotope effect anticipated for the cyclic mechanism. Although no similar data are available for other glycosides, the cyclic mechanism has been assumed to be generally applicable.

A half-chair intermediate, shown in Fig. 3, in the cyclic hydrolysis mechanism has been proposed by Edward (7). This somewhat flattened carbonium ion was postulated to serve as the common intermediate in the hydrolysis of $\alpha$- and $\beta$-glycosides. This intermediate is thought to be formed by rotation about the carbon-carbon bonds between $C_2$ and $C_3$ and also between $C_4$ and $C_5$. The ease with which this intermediate may be adopted will depend on the conformational stability of the original glycoside.

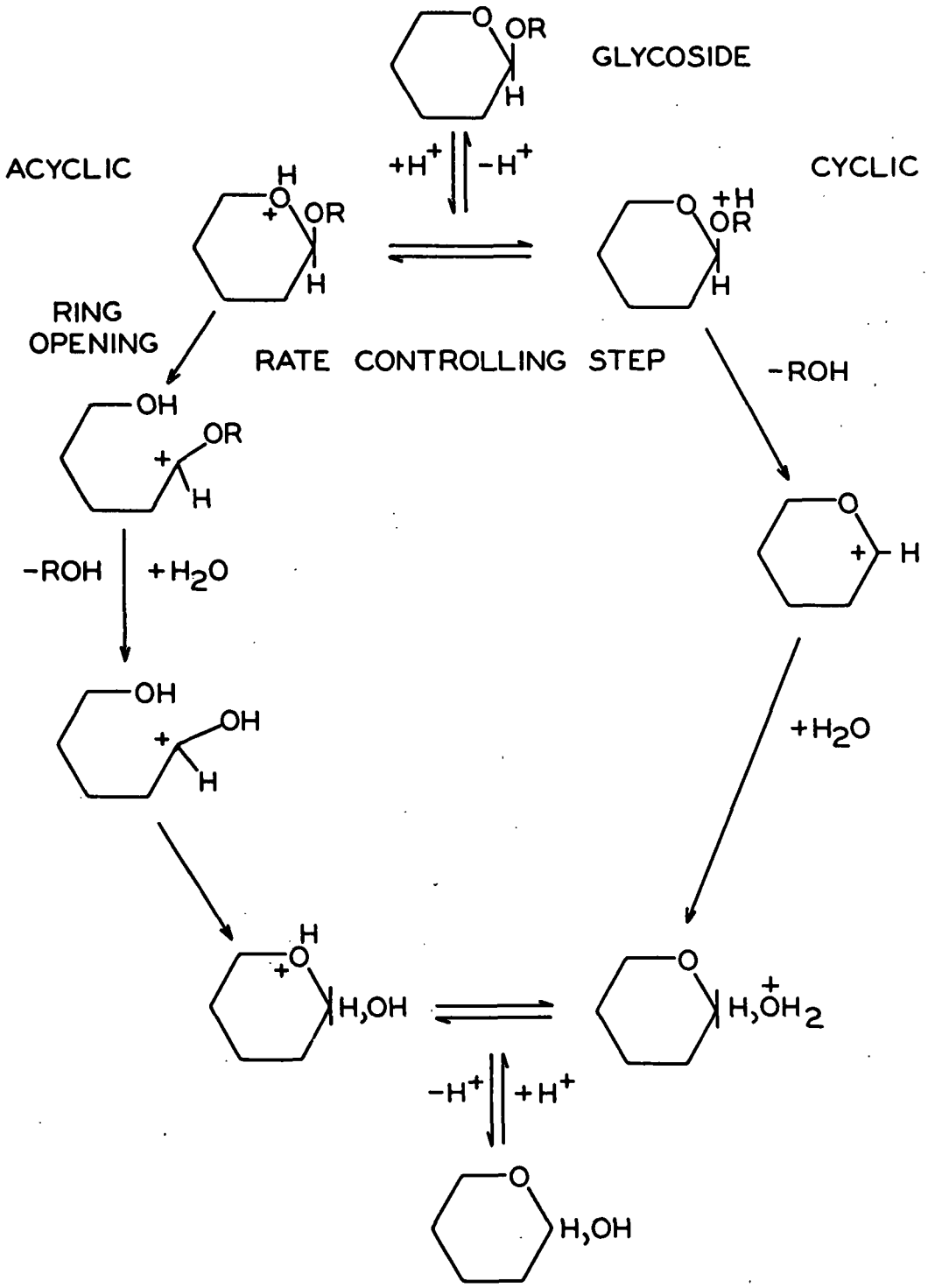



Figure 2. Postulated Hydrolysis Mechanisms (1, 2, 4, 6)

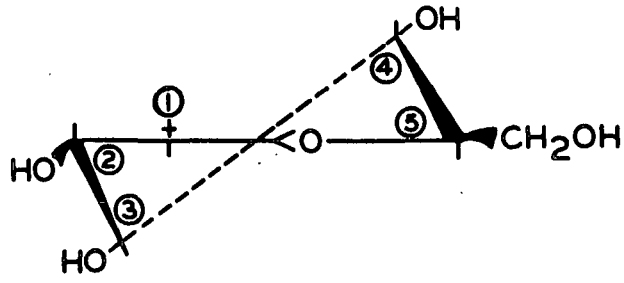



Figure 3. Glucose Carbonium Ion in the Pyranose Half-Chair Conformation (7)

## MODIFYING INFLUENCES

### Conformation

The C1 chair form has been shown by Reeves (8) to be the most stable conformation, of the eight possible chair and boat conformations, for the glucopyranoside ring. This particular conformation was a result of nonbonding interactions between the substituents of the basic pyran ring. (It should be noted that all future references to substitution will be made in terms of the six-member pyran ring. As an example, glucose is a $C_5$ substituted compound.) These groups (consisting of the aglycon; the $C_5$ hydroxymethyl group; the $C_2$, $C_3$, and $C_4$ hydroxyls; and five hydrogens) restrict rotation about the carbon-carbon bonds and hence the conformation is stabilized. The conformational stability of a particular glycoside will affect the ease with which the half-chair intermediate may be adopted and will thus affect the hydrolysis reaction rate. Variations in the substitution of the basic pyran ring will, therefore, alter the rate of glycoside hydrolysis. The order of stability to acid hydrolysis of the following glycosides reflects the effect of substituent size on the reaction rate: heptopyranosides > hexopyranosides > 6-deoxyhexopyranosides > pentopyranosides. Similarly, methyl

2- and 3-deoxypyranosides are more reactive than the corresponding methyl pyranosides (2).

Figure 4 shows methyl β-D-glucopyranoside in the familiar C1 conformation, using both the Reeves (8) and Newman (9) notations.

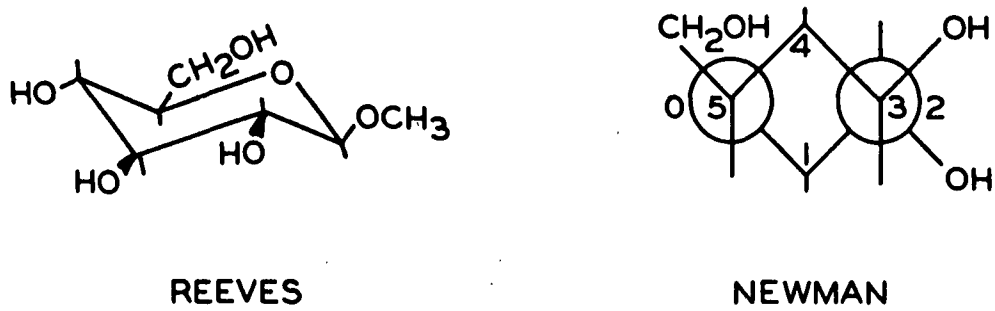



Figure 4. Glucopyranoside Notations for C1 Conformation

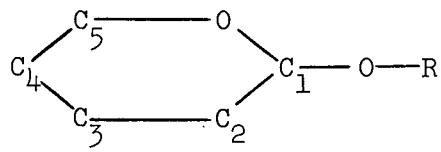
Substitution

Although many types of substitution affect the rate of acid hydrolysis, only two particular aspects will be considered: (1) the influence of the carboxyl group, and (2) the influence of "macro" substitution, i.e., the introduction of an entire sugar molecule. Both of these topics bear directly on the hydrolysis of the 4-O-methylglucuronoxylan polymer.

It has often been observed that the uronic acid glycosides are more stable to acid hydrolysis than the analogous neutral glycosides. This effect is usually attributed directly to the carboxyl group. The nature of this stabilizing influence has been investigated on a limited scale. Dyer, et al. (10) have reported a ninefold decrease in the rate of hydrolysis of the analogous 1-O-methyltetrahydropyran*,

*The conventional nomenclature of the tetrahydropyran ring has been ignored to emphasize the analogy to the pyranose ring. 1-O-Methyl tetrahydropyran is actually 2-methoxy-tetrahydropyran by the usual convention. It may also be thought of as the methyl glycoside of 2,3,4-deoxy-D-xylopyranose.

following introduction of a carboxyl group at $C_5$. Dyer, et al., have attributed this increased stability to changes in the electron density of the glycosidic linkage, $C_1$—O—R in Fig. 5, brought about by the inductive influence of the electrophilic carboxyl group. Marchessault and Rånby (11), Whistler and Richards (12), and Hamilton and Thompson (13) have advanced similar hypotheses to explain the stability of glycosidic linkages adjacent to carboxyl substitution in 4-O-methylglucuronoxylans. Several investigations using model compounds (14-17) have been undertaken to test the induction stabilization hypothesis, but no clear picture has yet emerged.




C = carbon
O = oxygen
R = aglycon

Figure 5. Numbering System for the Carbon Atoms of the Pyranoside Ring

The introduction of a sugar unit at either $C_2$ or $C_5$ of the pyranoside ring appears to affect the hydrolysis of the glycosidic linkage at $C_1$. Such substitution is common in the naturally occurring polysaccharides. The influence of this substitution may be noted in the structure of the oligosaccharide fragments occurring in the acid hydrolyzate of several polysaccharides.

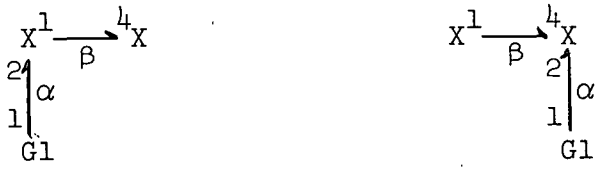
Hamilton and Thompson (18), as well as many others, have isolated the common aldotriouronic acid, (I) in Fig. 6, from the acid hydrolyzate of a 4-O-methylglucuronoxylan. However, these workers were unable to find any evidence for the existence of the isomeric aldotriouronic acid, (II) in Fig. 6, which might have been expected to occur on the basis of the structure of the starting material.

(I) (II)

X = xylopyranose unit
A = 4-O-methylglucuronic acid unit

Figure 6. Aldotriouronic Acids Expected from the Acid Hydrolyzate of a 4-O-Methylglucuronoxylan

McKee (19) has isolated the neutral trisaccharide, (III) in Fig. 7, from the acid hydrolyzate of a 4-O-methylglucoxylan, but he failed to find any significant evidence of the isomeric trisaccharide, (IV) in Fig. 7, which might have been expected to occur on the basis of the structure of the starting material.



(III) (IV)

X = xylopyranose unit
Gl = 4-O-methylglucopyranose unit

Figure 7. Neutral Trisaccharides of McKee (19)

Whistler and Durso (20) have isolated the neutral trisaccharide, (V) in Fig. 8, from the hydrolyzate of guaran. Contrary to expectations, these workers were unable to find the isomeric neutral trisaccharide, (VI) in Fig. 8.

(V) (VI)

Gal = galactopyranose unit
M = mannopyranose unit

Figure 8. Neutral Trisaccharides of Whistler and Durso (20)

The conformation stabilization effect, suggested by McKee (19), may be responsible for the preferential stability of one isomer in each of the above cases. Substitution of a bulky group at $C_2$, as in the molecules illustrated in Fig. 6 and 7, or at $C_5$, as in the molecule illustrated in Fig. 8, should restrict rotation about the carbon-carbon bonds ($C_2$–$C_3$ and $C_4$–$C_5$ as numbered in Fig. 5, p. 9) in the substituted unit. Thus, formation of the half-chair intermediate would be hindered. This restriction would, in turn, increase the stability of the glycosidic linkage at $C_1$ of the substituted unit. This increase in stability would account for the preferential formation of (I), (III), and (V), as discussed above.

Aglycon

The character of the aglycon may have considerable effect on the rate and mechanism of acid hydrolysis. Overend, Rees, and Sequeira (21) have demonstrated, as shown by the data in Table I, that the anomeric configuration and chemical nature of the aglycon may exert strong influence on the hydrolysis rate. However, introduction of and changes in polar substituents on the aglycon have been reported, by Semke (17) and Timell (22), to produce little change in the hydrolysis rate.

TABLE I

RELATIVE HYDROLYSIS RATES OF SEVERAL PYRANOSIDES IN 2.0N HCl AT 60°C. (21)

| Compound | Relative Rates |
|---|---|
| Methyl-α-D-glucopyranoside | 1.00 |
| Methyl-β-D-glucopyranoside | 1.78 |
| Methyl-α-D-xylopyranoside | 3.80 |
| Methyl-β-D-xylopyranoside | 8.32 |
| Phenyl-α-D-glucopyranoside | 53.7 |
| Phenyl-β-D-glucopyranoside | 13.2 |

## PAPER BIRCH 4-O-METHYLGLUCURONOXYLANS

Glucuronoxylans have been isolated from a variety of plant sources. Since comprehensive reviews (23-27) are available, only the paper birch glucuronoxylan will be discussed. Timell and co-workers (28-31), in a series of papers, have explored the chemical and physical characteristics of the paper birch 4-O-methylglucuronoxylan.

Glaudemans and Timell (29, 30) have isolated a 4-O-methylglucuronoxylan from a paper birch holocellulose. This polymer had a D.P. of 110-190, a xylose:4-O-methylglucuronic acid ratio of 11.4, and a high negative rotation. Acid hydrolysis, following methylation of this polymer, yielded 2-O- and 3-O-methylxylose (taken together), 2,3-di-O-methylxylose, 2,3,4-tri-O-methylxylose, and 2-O(2,3,4-tri-O-methylglucuronic acid)-3-O-methylxylose in a ratio of 3:116:1:11, respectively. On the basis of these data, and assuming that the minor amounts of monomethylxylose arose from demethylation of dimethylxylose, these workers suggested the structure illustrated in Fig. 9 for this polymer. This is the structure commonly advanced for 4-O-methylglucuronoxylans from other plant sources. The α-configuration of the 1,2 linkage between the acidic side chain F and the xylan backbone, has been confirmed by Gorin and Perlin (32).

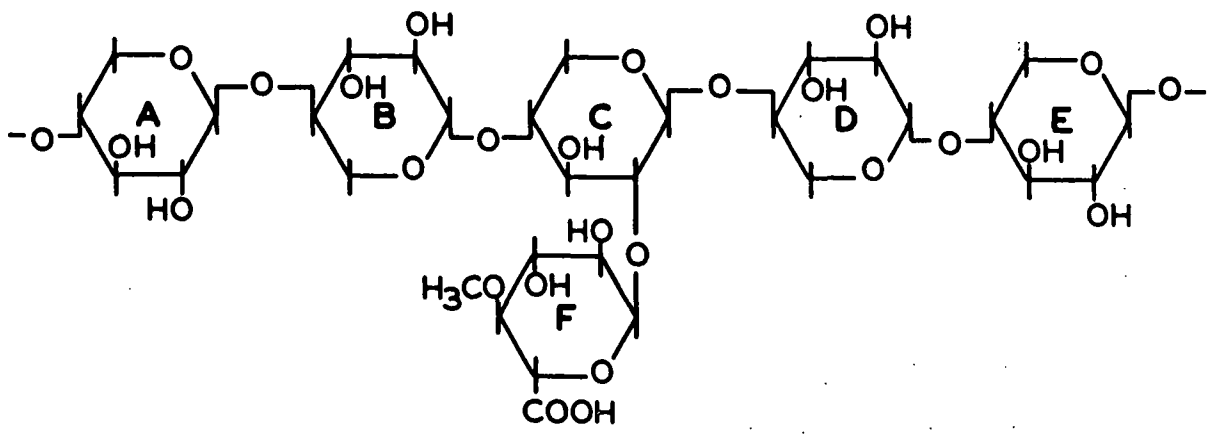



Figure 9. Segment of the Structure of the Paper Birch 4-O-Methylglucuronoxylan

Timell (31) has further supported the structure of Fig. 9 by studying the enzymatic hydrolysis of the paper birch glucuronoxylan. A series of acidic oligosaccharides, depicted in Fig. 10, were isolated from the reaction mixture. All these fragments were identified through the usual methylation techniques.

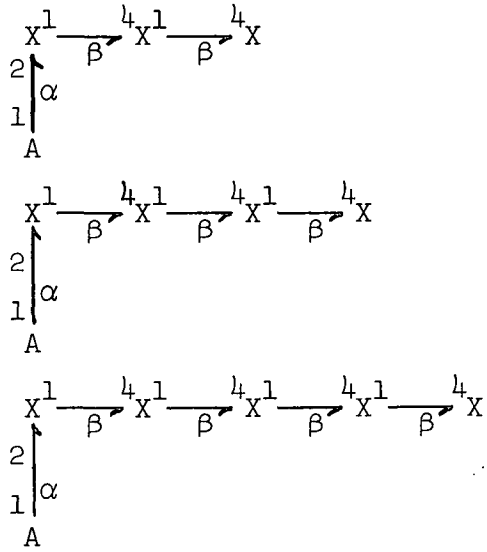



Figure 10. Timell's Oligouronic Acids (31)

## ANALYSIS OF THE PROBLEM

Previous investigations of the hydrolysis of 4-O-methylglucuronoxylans, as discussed above, have revealed a curious "anomaly." Although the structure of the parent polysaccharide is generally accepted as that illustrated in Fig. 9, no instance has been reported of an oligosaccharide fragment in which the linkage BC (in Fig. 9) persisted. This observation has led Marchessault and Rånby (11) to suggest that linkage BC may have been activated by the proximity of the acidic side chain. The mechanism proposed by these workers involved an extension of the induction stabilization theory, used to account for the stability of linkage CD in Fig. 9. Recent work by Johansson, Lindberg, and Theander (33) may be interpreted to suggest that, in fact, there may be little difference in the

stability of linkage BC (in Fig. 9) and the stability of the normal xylose-xylose β-1,4 glycosidic linkage, e.g., linkage AB or DE. These workers have determined rate constants for the acid-catalyzed hydrolysis of the three compounds depicted in Fig. 11. At all temperatures considered, the rate constants of (VII) and (VIII) were almost identical, while the rate constant of (IX) was an order of magnitude lower. This evidence is not consistent with the theory advanced by Marchessault and Rånby (11). The activation concept would predict a significant difference in the rate constants for all three sugars, with (VIII) being the highest and (IX) being the lowest.

(VII) $G^{1}\xrightarrow[\beta]{}{}^{4}G$

(VIII) $G^{1}\xrightarrow[\beta]{}{}^{4}GA$

(IX) $GA^{1}\xrightarrow[\beta]{}{}^{4}G$

G = glucupyranose unit
GA = glucuronic acid unit

Figure 11. Disaccharides Studied by Johansson, et al.

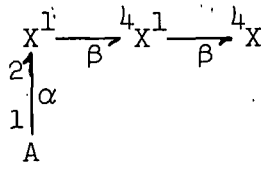
The apparent lability of linkage BC (in Fig. 9) may be accounted for by the conformation stabilization hypothesis of McKee and Dickey (19). In this interpretation, it is not the weakness of linkage BC but rather the stability of linkage CD (in Fig. 9) which is responsible for the aforementioned "anomaly." Thus, linkage BC may be no less stable than AB or DE, but being significantly less stable than CD, BC does not survive in the isolated trisaccharide fragment. However, an acidic fragment of higher DP might be expected to contain linkage BC as well as CD.

## STATEMENT OF THE PROBLEM

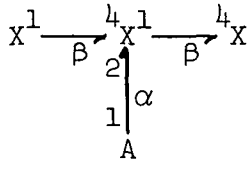
Two possible aldotriouronic acid isomers may be anticipated from the hydrolyzate of a 4-O-methylglucuronoxylan, on the basis of its structure. Only one of these two isomers has been isolated, although the second has been sought (18). Marchessault and Rånby (11) have suggested that an increase in glycosidic bond strength, due to the inductive influence of the carboxyl group, is responsible for the stability of one of these isomers. McKee and Dickey (19) have attributed this stability to steric factors, arising from the size of the uronic acid unit. If the tetrasaccharide acid fraction can be isolated, from the hydrolyzate of a 4-O-methylglucuronoxylan, and characterized, these two divergent hypotheses may be evaluated.

Three possible aldotetraouronic acid isomers, shown in Fig. 12, might arise from the partial hydrolyzate of a 4-O-methylglucuronoxylan, on the basis of its structure. The two theories used to account for the stability of the aldotriouronic acid, may be extended to the tetrasaccharide acid, in order to predict which of these isomers will occur. The induction stabilization theory of Marchessault and Rånby (11) predicts that only Isomer A will occur. Both Isomer B and Isomer C contain a linkage (BC in Fig. 9, p. 12) which these workers have suggested will be activated by the inductive influence of the carboxyl group. The conformation stabilization theory of McKee and Dickey (19) predicts similar quantities of Isomer A and Isomer B. Both of these isomers contain a linkage (CD in Fig. 9) which McKee suggests has been stabilized. Neither of these two hypotheses would predict an appreciable quantity of Isomer C.

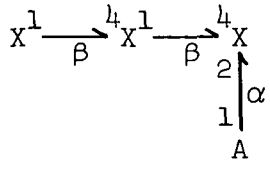
Isomer A



Isomer B



Isomer C



X = xylopyranose unit
A = 4-O-methylglucuronic acid unit

Figure 12. Possible Aldotetraouronic Acids from a 4-O-Methylglucuronoxylan

The specific objectives of this work were:

1. Isolation of the tetrasaccharide acid fraction from the partial acid hydrolyzate of a paper birch 4-O-methylglucuronoxylan;

2. Determination of the structure, or structures, of these fragments;

3. Investigation of the relative stability to acid hydrolysis of the glycosidic linkages in these fragments.

## EXPERIMENTAL RESULTS

Paper birch was chosen because of the consistent body of information available concerning the associated 4-O-methylglucuronoxylan (28-31). A tetrasaccharide acid (Isomer A) has also been isolated from the enzymatic hydrolysis of this polymer (34). However, acid hydrolysis will not necessarily yield the same isomer as enzyme hydrolysis.

### ISOLATION OF A 4-O-METHYLGLUCURONOXYLAN

A sample of airdry paper birch wood meal, 2657 g. (o.d.), was extracted with 25 liters of 0.1N sodium hydroxide for four hours in order to remove soluble lignin (35) and pectic materials (36). The sodium hydroxide solution was removed by filtration, neutralized, and retained for some future study. The birch meal residue was then extracted for two hours with 25 liters of ten per cent potassium hydroxide. The solution was removed by filtration, neutralized with acetic acid, and poured into ten gallons of 95% ethanol. This mixture was stirred vigorously and then allowed to settle overnight. Approximately seven gallons of supernatant were decanted and discarded. Ten gallons of fresh absolute ethanol were added and the mixture stirred. The suspension was then allowed to stand for 24 hours to allow the hemicellulose precipitate to settle. The hemicellulose was isolated by filtration on a table-top Buchner funnel; yield: 393 g. (o.d.); ash: 12.2% as $KC_2H_3O_2$; corrected yield: 345 g. (o.d.). The sample was retained as the moist filter cake.

A 13.2 g. (o.d.) sample of the 4-O-methylglucuronoxylan was placed in cellophane dialysis tubing and dialyzed against deionized and then distilled water over a two-week period. The product was concentrated and freeze dried;

yield: 12.0 g. Analysis: 3.97% moisture; 1.55% ash as potassium (IPC Procedure No. 4); 10.40% uronic anhydride (IPC Procedure No. 25); 1.78% methoxyl (IPC Procedure No. 18); intrinsic viscosity $[\eta] = 0.50$ in dimethylsulfoxide (Cannon No. 100 viscometer).

## GRADED ACID HYDROLYSIS

A 100 g. (o.d.) sample of the crude 4-O-methylglucuronoxylan was stirred overnight at room temperature in one liter of distilled water. The suspension was then heated to 70°C. and one liter of 2N sulfuric acid, at 70°C., was added. The reaction was maintained at 70°C. ± 1°C., with stirring, for fourteen hours. After cooling in a cold water bath, the hydrolyzate was neutralized to pH 6 with barium hydroxide solution. The reaction mixture was then centrifuged and the clear supernatant concentrated to a heavy sirup; approximate yield: 40 g.

## CRUDE ALDOTETRAOURONIC ACID

### ISOLATION

The sirup resulting from the graded acid hydrolysis was deionized with Amberlite IR-120 ($H^+$) cation-exchange resin*. Two grams of material, calculated as the barium uronate, were placed on each of twenty Whatman No. 17 sheets, as described in Appendix I. The sheets were then eluted for three days with Solvent B. Six fractions were noted, corresponding to xylose, aldobiouronic acid, xylobiose, aldotriouronic acid, xylotriose-aldotetraouronic acid, and a mixture of the higher oligosaccharides. The xylotriose-aldotetraouronic acid fraction was isolated and the aldotetraouronic acid purified by further paper chromatography

*Amberlite ion-exchange resins are manufactured by Rohm and Haas, Philadelphia.

on water-washed Whatman No. 17 sheets. The entire fraction, approximately two grams of a heavy sirup, was placed on a single 18-1/2 by 22-1/2 inch sheet and eluted serially with Solvents B and C, three days with each; aldotetraouronic acid yield: 1.01 g. of dried sirup. Specific rotation $[\alpha]_D^{25}$ = 23° (c, 0.667 in water); 4.8% methoxyl (IPC Procedure No. 18).

CHARACTERIZATION

The tetrasaccharide acid, isolated above, formed the third member of an acidic homologous series, as shown in Fig. 13. The first two members of this series, the aldobiouronic acid and the aldotriouronic acid, are well known. The chromatographic mobilities were identical with those of authentic samples. The crystalline aldotriouronic acid was isolated and identified through its melting point, 180°C., and IR spectrum, which was identical to that of an authentic sample.

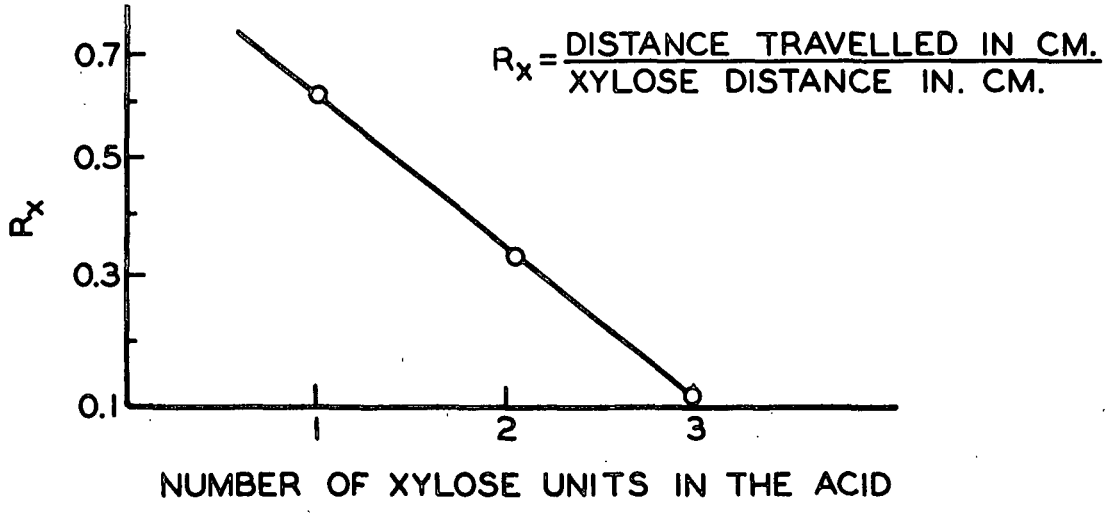



Figure 13. The Acidic Polymer Homologous Series from Paper Birch

The tetrasaccharide acid appeared to be homogeneous by paper chromatography in Solvents A, B, and C. After several months as a dried sirup and in the acid form, the tetrasaccharide acid became contaminated with xylose and the aldotriouronic acid. In order to prevent this apparent autohydrolysis, the aldotetraouronic acid was kept in the barium salt form for most purposes. Paper electrophoresis, as described in Appendix II, in 0.1M sodium borate buffer for three hours at 500 volts, showed that the aldotetraouronic acid consisted of three components.

FRACTIONATION

A 460-mg. sample of the aldotetraouronic acid was placed on a seven-inch strip of Whatman No. 17 paper. The sheet was saturated with 0.1M sodium borate and placed in the electrophoresis chamber, as described in Appendix II. A 360-volt potential was applied for five hours. Three fractions, as shown in Table II, were located and isolated from the preparative electrophoretogram in the usual manner.

TABLE II

PREPARATIVE ELECTROPHORESIS FRACTIONS

| Fraction | $M_g$[a] | Yield, mg. | Yield, % (of recovered) | Equivalent Weight |
|---|---|---|---|---|
| (I) | 0.67 | 49 | 11 | 560 |
| (II) | 0.56 | 386 | 86 | 604 |
| (III) | 0.13 | 13 | 3 | 1370 |
| Total recovered | | 448 mg. | 100% | |
| Total applied | | 460 mg. | | |
| Per cent recovered | | 97.5% | | |

[a] $M_g$ = Electrophoretic migration with respect to glucose ($M_g$ = 1.00).

The equivalent weight values, also shown in Table II, were determined by potentiometric titration of each sample with 0.0962N sodium hydroxide. The end point appeared to occur at pH 8.0. The equivalent weight of Fraction (II) compares very favorably with the theoretical value of 604.5 atomic weight units for an aldotetraouronic acid. The specific rotation was $[\alpha]_D^{25} = 24°$ (c, 0.800 in water).

No attempt was made to determine the identity of either Fraction (I) or (III), although it is possible that Fraction (I) is also a tetrasaccharide acid.

## STRUCTURAL INVESTIGATION OF PURIFIED ALDOTETRAOURONIC ACID

### METHYLATION (34)

A 150-mg. sample of Fraction (II) was converted to the nonreducing tetrasaccharide acid by reduction in 25 ml. of four per cent sodium borohydride — 0.1N sodium hydroxide solution. The reaction mixture was neutralized with dilute acetic acid, deionized on Amberlite IR-120 ($H^+$), and concentrated, under reduced pressure, to dryness several times with methanol. This procedure served to remove the borate residue.

The sirupy, nonreducing tetrasaccharide acid was dissolved in 6.25 ml. of distilled water containing 300 mg. of sodium bicarbonate. After flushing the reaction vessel thoroughly with nitrogen, 3.25 ml. of dimethyl sulfate and 3.25 ml. of 40% sodium hydroxide solution were added, dropwise and alternately, over a four-hour period. The reaction vessel was maintained under a slight positive nitrogen pressure. After stirring for one hour, 1.8 g. of solid sodium hydroxide were added and 3.25 ml. of dimethyl sulfate were added dropwise over 18 hours. A

second addition of solid sodium hydroxide (1.8 g.) and dimethyl sulfate (3.25 ml.) was completed in the same manner. The reaction mixture was stirred overnight. The solution was then neutralized to pH 5 with N sulfuric acid and extracted with chloroform. The chloroform extract was washed twice with distilled water and concentrated to a heavy sirup; yield: 215 mg. of the dried sirup. The product represents 117% of the starting material. This high yield may have been due to inorganic salts and sodium methylate which are also products of this reaction. Residual moisture may have increased the yield also.

The entire sample was dissolved in 5 ml. of dry dimethylformamide. Two milliliters of methyl iodide and 250 mg. of Drierite (anhydrous calcium sulfate) were added. The mixture was allowed to stir for four hours. Two grams of silver oxide were added in small portions over a three-hour period. A second addition of methyl iodide and silver oxide was made in the same manner and the reaction mixture stirred for three days. The solids were then removed by filtration on a Celite pad and the residue washed extensively with chloroform. The combined filtrates were evaporated to 5 ml. and added to 50 ml. of chloroform. The chloroform was then extracted with 25 ml. of 10% potassium cyanide solution followed successively by two water washings. The chloroform solution was then concentrated to a heavy sirup and dried in a moving air stream; yield: 176 mg. or 97% of theoretical. Infrared spectra revealed no significant peak in the hydroxyl stretching region at 2.85 microns, although the experimental value of 46% methoxyl was below the theoretical 49.7%.*

REDUCTION (34)

A sample of 160 mg. of the fully methylated, nonreducing Fraction (II) was dissolved in 9.5 ml. of dry tetrahydrofuran. Lithium aluminum hydride (160 mg.)

*The validity of this criterion for complete methylation is discussed in Appendix III.

was added slowly. The reaction mixture was stirred for two hours. Following completion of the reduction, the excess lithium aluminum hydride was destroyed by gradual addition of ethyl acetate to the cold reaction mixture. Distilled water was added and the product filtered and deionized on Amberlite IR-120 ($H^+$) and IR-45 ($OH^-$). The product was concentrated to a thick sirup under reduced pressure.

METHANOLYSIS

The fully methylated and reduced Fraction (II) was dissolved in 25 ml. of 0.5N methanolic hydrogen chloride. The solution was refluxed for eight hours, neutralized with silver carbonate, and filtered. The filtrate was deionized with Amberlite IR-120 ($H^+$) and IR-45 ($OH^-$), concentrated to dryness under reduced pressure, and dissolved in 1 ml. of ethanol.

GAS CHROMATOGRAPHIC ANALYSIS

The Aerograph Model A 600-B Gas Chromatograph, described in Appendix IV, was used to analyze the methanolysis mixture. The four foot, butanediol succinate polyester column was maintained at 160°C. and the nitrogen flow rate at 20 ml. per minute. A sample was injected through the sample port and starting time noted on the output trace. The instrument was allowed to operate for 30 minutes after the last component was noted on the output trace. Figure 14 illustrates the instrument output. Nine different peaks were noted. The retention times and peak areas were measured and these data are recorded in Table III.

Tentative identification of these nine peaks was made by comparison of retention times with those of authentic samples. These identifications were supported by later work. The assignment of the anomeric configuration was made by use of the

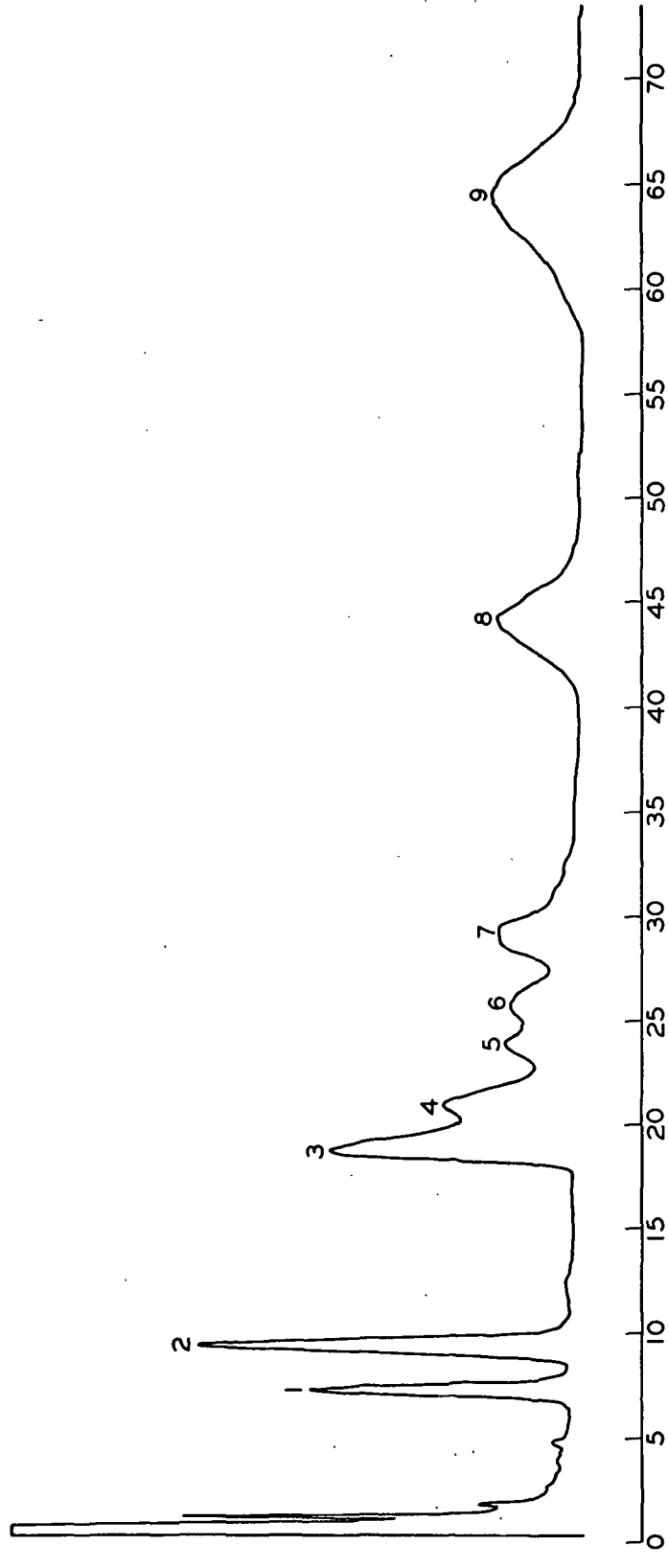



Figure 14. Gas Chromatogram of Methanolysis Mixture (the Numbered Peaks are Identified in Table III)

TABLE III

GAS CHROMATOGRAPHIC ANALYSIS AND ISOLATION DATA

| Peak | Compound | $T_r$ | $A_r$ | $C_f$ | $M_1$ | I | F | $M_2$ |
|---|---|---|---|---|---|---|---|---|
| 1 | Methyl 2,3,4-tri-O-methyl-β-xylopyranoside | 7.2 | 25.6 | 1.00 | 0.98 | 1 | 5.5 | 0.93 |
| 2 | Methyl 2,3,4-tri-O-methyl-α-xylopyranoside | 9.4 | 43.7 | | | 2 | 11.0 | |
| 3 | 1,2,3,5-tetra-O-methyl-xylitol | 18.8 | 63.7 | 1.16 | 1.04 | 3 | 17.5 | 0.97 |
| 4 | Methyl 3,4-di-O-methyl-β-xylopyranoside | 20.9 | 34.3 | 1.22 | 0.98 | 4 | 2.0[a] | 0.06 |
| 6 | Methyl 3,4-di-O-methyl-α-xylopyranoside | 25.7 | 22.3 | | | | | |
| 5 | Methyl 2,3-di-O-methyl-β-xylopyranoside | 23.8 | 23.0 | 1.22 | 1.01 | | | 0.06 |
| 7 | Methyl 2,3-di-O-methyl-α-xylopyranoside | 29.0 | 35.5 | | | | | |
| 8 | Methyl 3-O-methyl-α,β-xylopyranoside | 44.0 | 45.4 | 1.44 | 0.92 | 5 | 13.5 | 0.88 |
| 9 | Methyl 2,3,4-tri-O-methyl α,β-glucopyranoside | 64.5 | 74.8 | 0.95 | 1.00 | 6 | 20.5 | 1.00 |

$T_r$ = retention time in minutes; $A_r$ = peak area in square units; $C_f$ = calibration factor; $M_1$ = mole ratio calculated from $A_r$; I = isolated fraction number; F = weight in mg. of I; $M_2$ = mole ratio calculated from F. $M_1$ and $M_2$ were both based on peak 9 M = 1.00.

[a] Assumed to be divided equally between the two dimethyl xyloses.

general rule proposed by Bishop (37). The anomer having the lowest retention time will be that one having the trans relationship between the $C_1$ and $C_2$ methoxyl groups. This generalization appears to be valid for methylated derivatives of D-glucose, D-xylose, D-galactose, and D-mannose.

The Aerograph A600-B was calibrated, as described in Appendix IV, with authentic samples of 2,3,4-tri-O-methylxylose; 1,2,3,5-tetra-O-methylxylitol; 3-O-methylxylose; and 2,3,4-tri-O-methylglucose. A calibration factor for the two di-O-methylxyloses could not be determined, due to peak overlap. A value of $C_f$ = 1.22 was assumed, however, by averaging the calibration factors for the mono- and tri-O-methylxylose fractions. The calibration factor was necessary because the flame detector does not exhibit the same response for all the methylated compounds under consideration. The calibration factors, shown in Table III, were used to correct the instrument response, so that all peak areas were comparable. The apparent mole ratios, calculated from peak areas, are also shown in Table III.

Approximately 85%, supposed to represent 135 mg. of the fully methylated tetrasaccharide acid (of the methanolysis mixture), was separated into six fractions by use of the Aerograph Model A 90-s Gas Chromatograph, as described in Appendix IV. Four peaks, noted in Table III as 4,5,6, and 7, were collected as one fraction due to peak overlap. The other peaks were collected as separate fractions. All six fractions were dissolved in ethyl ether, filtered, dried, and weighed. The fraction weights and corresponding mole ratios appear in Table III. The total weight, 70.0 mg., of the isolated fractions represented only 54 mole per cent of the starting tetrasaccharide acid. This was a significant loss of material and represents a weakness in the quantitative data. There is some possibility that incomplete methanolysis was responsible for this material

loss. The methanolysis procedure used was that recommended by Timell (34) and is usually assumed to be complete. It was assumed, therefore, that material losses occurred randomly and that the material isolated was representative of the purified aldotetraouronic acid fraction [Fraction (II)].

## IDENTIFICATION OF METHYLATED COMPOUNDS

Each of the six collected fractions was dissolved in 2 ml. of N sulfuric acid and sealed in a 4-ml. vial. Following hydrolysis for 1-1/2 hours at 105°C., the solutions were neutralized with barium carbonate and centrifuged. The clear supernatant was extracted three times with chloroform. The chloroform solution was evaporated to dryness and dissolved in ether. The fractions were examined by paper chromatography in Solvent D by comparison with authentic samples. The identifications made in Table III appeared to be confirmed. ($R_x$ values are noted under the summary for individual compounds.) Fraction 3 was nonreducing, even after hydrolysis. Fraction 4 appeared to be at least one dimethylxylose, but no further identification was possible.

The four main fractions were identified with reasonable certainty. (All IR spectra are shown in Fig. 15.)

### 2,3,4-Tri-O-methylxylose

Fractions 1 and 2 were combined and recrystallized once from ethyl ether; m.p. 89-90°C.; literature m.p. 91-92°C. (38). The IR spectrum was identical to that of an authentic sample of 2,3,4-tri-O-methyl-D-xylose. Paper chromatographic $R_x$ in Solvent D = 23.0.

### 1,2,3,5-Tetra-O-methylxylitol

Fraction 3 was a mobile, nonreducing sirup. Identification was made through the p-nitrobenzoate derivative (39). The sirup was dissolved in dry pyridine and

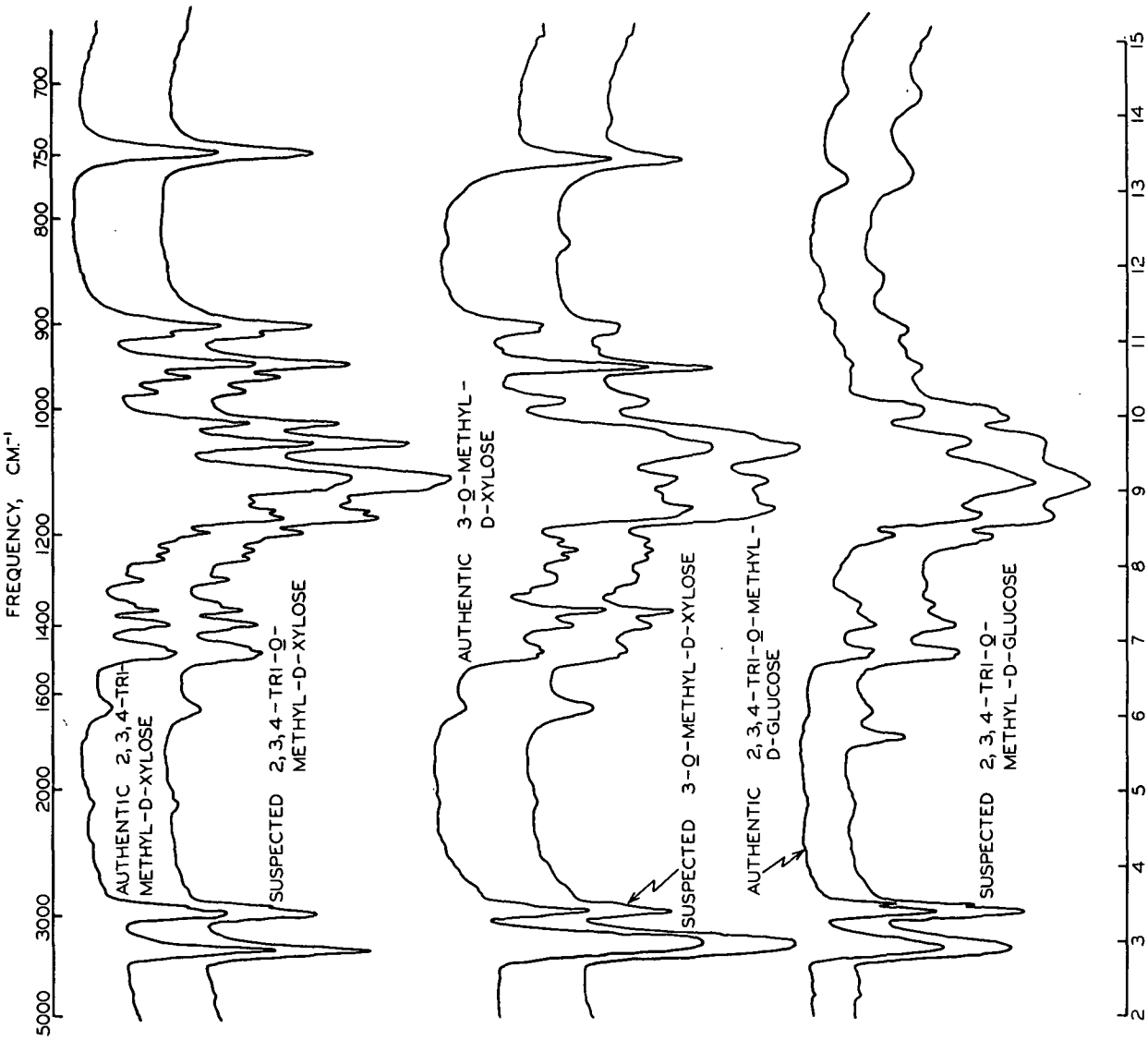



Figure 15. IR Spectral Identifications

35 mg. of recrystallized p-nitrobenzoyl chloride (m.p. 71-72°C.) were added. The mixture was heated in a sealed vial at 65°C. for 40 minutes. After standing at room temperature for 24 hours, the solution was neutralized with saturated sodium bicarbonate solution and extracted three times with chloroform. The chloroform solution was dried overnight over anhydrous sodium sulfate and then allowed to evaporate to dryness. The crystalline material was collected and washed in cold methanol; m.p. 185-186°C.; literature m.p. 187-189°C. (39).

3-O-Methylxylose

Fraction 5 was recrystallized once from ethyl acetate; m.p. 101-102°C.; literature m.p. 102-103°C. (38). The IR spectrum was identical to that of an authentic sample of 3-O-methyl-D-xylose. The paper chromatographic $R_X$ in Solvent D was 4.92.

2,3,4-Tri-O-methylglucose

Fraction 6 was a mobile, reducing sirup. The IR spectrum was identical to that of an authentic sample of 2,3,4-tri-O-methyl-D-glucose. (A slight carbonyl peak at 5.7 microns was attributed to impurities arising from the gas chromatography column substrate.) The paper chromatographic $R_X$ in Solvent D was 22.3.

## MILD ACID HYDROLYSIS OF FRACTION (II)

A 50-mg. sample of the purified aldotetraouronic acid, Fraction (II), was hydrolyzed for one hour at 70°C. in 25 ml. of 0.5N sulfuric acid. The reaction mixture was neutralized with barium carbonate, centrifuged, and the clear supernatant concentrated to a thin sirup. Examination by paper chromatography revealed, on the basis of $R_X$ and spot size, a 1:1 ratio of xylose and an aldotriouronic acid, as well as residual aldotetraouronic acid.

A 50-mg. sample of the purified aldotetraouronic acid, Fraction (II), was dissolved in a solution of 4% sodium borohydride – 0.1N sodium hydroxide. After two hours at room temperature, the solution was neutralized with dilute acetic acid, deionized with IR-120 ($H^+$), and concentrated to dryness. Methanol was added three times and the solution evaporated to dryness. The resulting sirup, the nonreducing tetrasaccharide acid, was hydrolyzed by the conditions described above. Paper chromatographic analysis revealed xylose as the only reducing compound. A nonreducing trisaccharide acid and unchanged starting material were also noted, on treatment of the chromatogram with Spray C. No xylitol spot was noted, indicating that less than one microgram was present.

These two hydrolysis experiments appear to be consistent only with the symmetric isomer of the aldotetraouronic acid, Isomer B in Fig. 12. Figure 16 illustrates the reaction sequence in these two hydrolyses. Neither of the other two isomers would have given hydrolysis products consistent with both of these experiments.

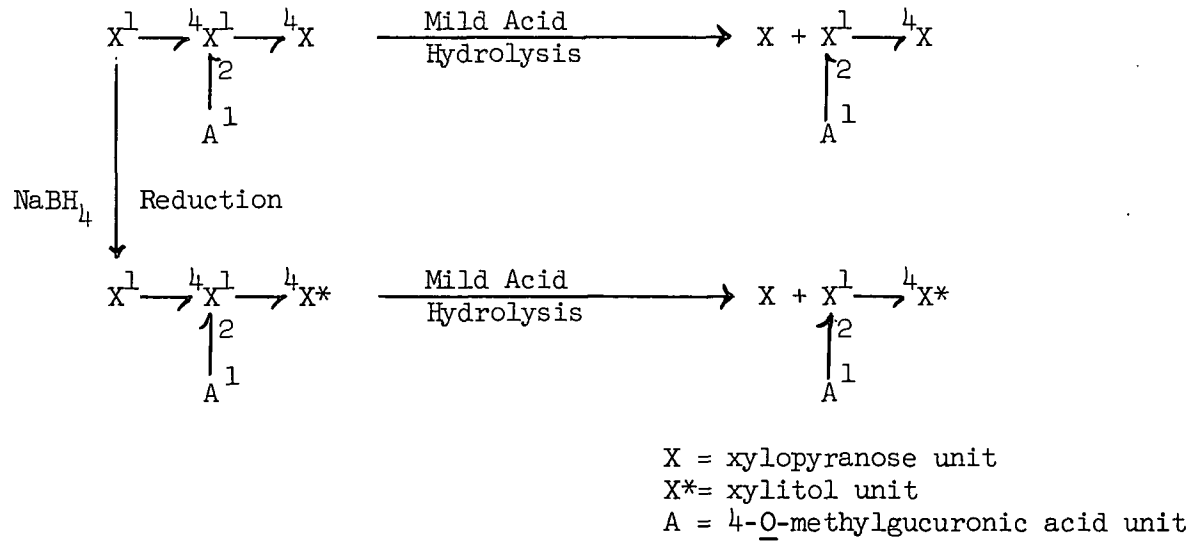
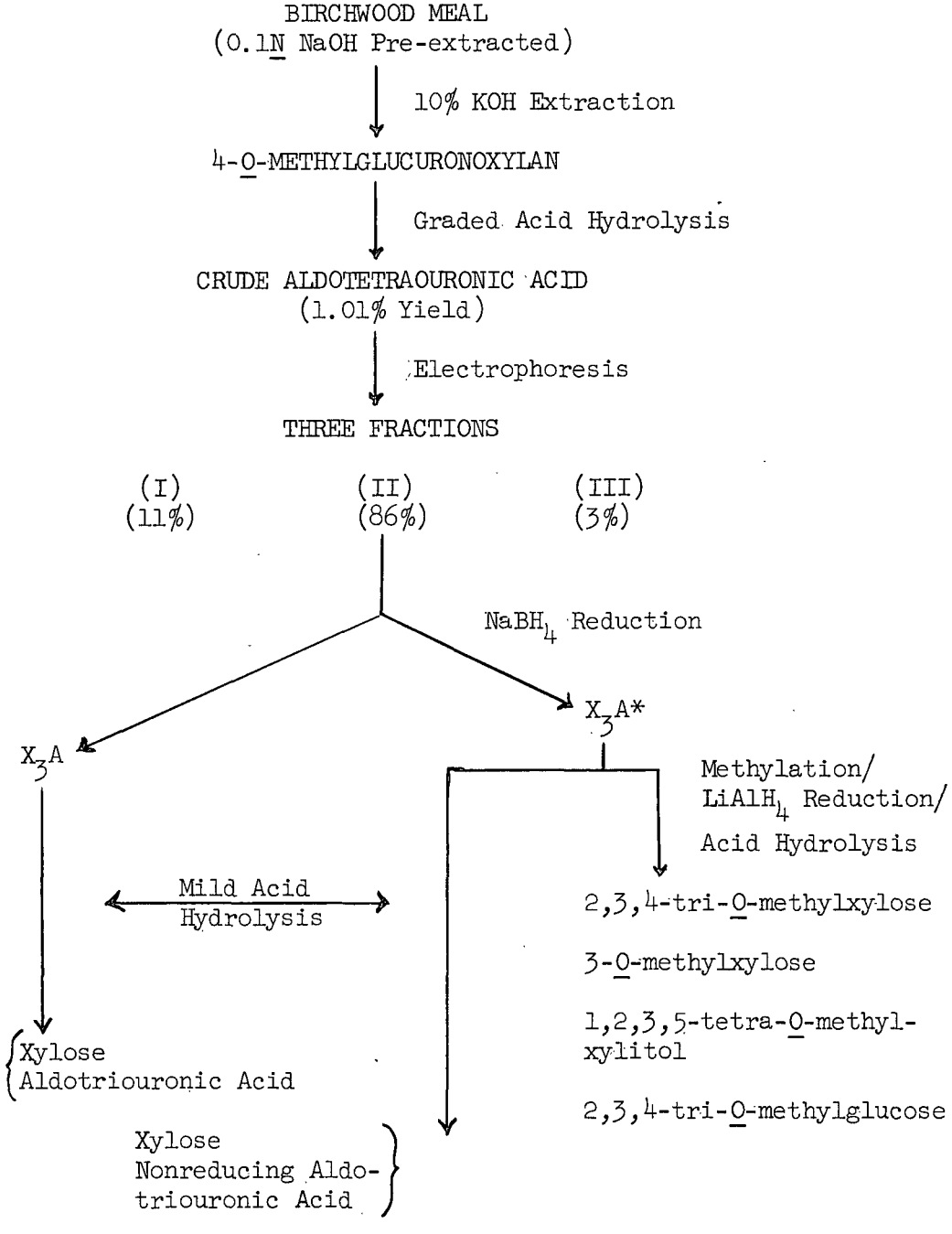



Figure 16. Acid Hydrolysis of the Symmetric Reducing and Nonreducing Aldotetraouronic Acids

FLOW CHART SUMMARIZING EXPERIMENTAL WORK




($X_3A$ = aldotetraouronic acid; $X_3A^*$ = nonreducing aldotetraouronic acid)

# DISCUSSION OF RESULTS

## STRUCTURE OF THE PURIFIED ALDOTETRAOURONIC ACID

An aldotetraouronic acid fraction was isolated by paper chromatography and paper electrophoresis from the partial hydrolyzate of a paper birch 4-O-methylglucuronoxylan. This acid was characterized by specific rotation, $[\alpha]_D^{25} = 24°$; equivalent weight = 604; $R_x = 0.14$ (Solvent A); and $M_g = 0.56$ (0.1M sodium borate buffer). Mild acid hydrolysis yielded xylose and a trisaccharide acid, presumably the common aldotriouronic acid. It is of interest to note that the specific rotation and paper chromatographic mobility values were similar to those of the tetrasaccharide acids isolated by Hamilton and Thompson (18) and Timell (34). The acid isolated by Hamilton a nd Thompson, from the partial hydrolyzate of a western hemlock 4-O-methylglucuronoxylan, was not investigated thoroughly, although it might be assumed to have been similar to the acid isolated in this work. The tetrasaccharide acid isolated by Timell, from the enzymatic hydrolyzate of a paper birch 4-O-methylglucuronoxylan, was shown to have been principally Isomer B. The similarity of the values of specific rotation and paper chromatographic mobility is not surprising, however, since both isomers have the same component sugars connected by the same glycosidic linkages.

As noted previously (p. 16), three possible tetrasaccharide acid isomers may be expected to occur in the hydrolyzate of a 4-O-methylglucuronoxylan. The purified aldotetraouronic acid fraction was studied through a reaction sequence involving sodium borohydride reduction, methylation, lithium aluminum hydride reduction, and methanolysis. This procedure (described in detail under the Experimental Results) was expected to unequivocally identify each of the three possible isomers, separately or in a mixture, by the distribution of the methylated

components present in the methanolysis mixture. The distribution of methylated components for all three isomers is shown in Table IV. Variations in the quantity of each methylated component should reflect the relative quantity of each of the three acids in an isomeric mixture.

TABLE IV

METHYLATED COMPONENTS FROM A MIXTURE OF ALDOTETRAOURONIC ACIDS

| Methylated Components | Isomers A | B | C | Mole Ratios $\underline{M}_1$ | $\underline{M}_2$ |
|---|---|---|---|---|---|
| 2,3-Di-O-methylxylose | X | | X | 0.98 | 0.06 |
| 3,4-Di-O-methylxylose | X | | | 1.01 | 0.06 |
| 3-O-Methylxylose | | X | | 0.92 | 0.88 |
| 2,3,4-Tri-O-methylxylose | | X | X | 0.98 | 0.93 |
| 1,3,5-Tri-O-methylxylitol | | | X | 0.00 | 0.00 |
| 1,2,3,5-Tetra-O-methylxylitol | X | X | | 1.04 | 0.97 |
| 2,3,4-Tri-O-methylglucose | X | X | X | 1.00 | 1.00 |

$\underline{M}_1$ = mole ratio calculated from gas chromatographic data; $\underline{M}_2$ = mole ratio calculated from isolation data; see Table III.

The mole ratio data of Table IV, regrettably, are not mutually consistent. The inconsistency arises in the gas chromatographic analysis data, $\underline{M}_1$. These data suggest, on the basis of the methylated components associated only with Isomer A or Isomer B, that approximately equimolar quantities of Isomer A and Isomer B were present in the aldotetraouronic acid starting material. However, these methylated components were not balanced by sufficient quantities of 1,2,3,5-tetra-O-methylxylitol and 2,3,4-tri-O-methylglucose, which would have been derived from both Isomer A and Isomer B. Thus, the gas chromatographic data caanot be literally interpreted on the basis of an aldotetraouronic acid starting material.

Two parallel interpretations of the gas chromatographic analysis data may be made, however, by accepting one of the following assumptions:

(a) The di-O-methylxylose peak areas were significant, whereas the mono- and tri-O-methylxylose peak areas were partially spurious; or

(b) The mono- and tri-O-methylxylose peak areas were significant whereas the di-O-methylxylose peak areas were partially spurious.

As previously discussed, each of the three possible tetrasaccharide acid isomers may be identified by the characteristic methylated components, arising from the reaction sequence described. Utilizing assumption (a), the data of $\underline{M}_1$ in Table IV show approximately equimolar quantities of the four components characteristic of Isomer A. In the first case, therefore, Isomer A would be the principal component of the purified aldotetraouronic acid fraction. Utilizing assumption (b), the data of $\underline{M}_1$ in Table IV show approximately equimolar quantities of the four components characteristic of Isomer B. In this second case, therefore, Isomer B would have been the principal component of the purified aldotetraouronic acid fraction. Acceptance of either assumption requires an explanation of the origin of the spurious peak areas.

The composition of the methanolysis mixture was further explored by isolation and investigation of the material corresponding to each peak in the gas chromatogram of Fig. 14 (p. 24). As noted in $\underline{M}_2$ of Table IV, the two di-O-methylxylose components represented only a small fraction, approximately six mole per cent, of the material isolated. Approximately equimolar quantities of 3-O-methylxylose and 2,3,4-tri-O-methylxylose were isolated as well as approximately equimolar quantities of 1,2,3,5-tetra-O-methylxylitol and 2,3,4-tri-O-methylglucose. These data indicated that the purified aldotetraouronic acid was

predominantly (over 90 mole per cent) Isomer B. Isomer A also appeared to have been present as a minor component. Unfortunately, the material isolated represented only 54 mole per cent of the starting tetrasaccharide acid fraction. This rather disappointing yield represented about 80% of that expected, on the basis of similar investigations by other workers (34, 39). It is not possible, therefore, to make an absolute determination of the isomeric composition of the purified aldotetraouronic acid fraction. The isolation data did indicate that a significant quantity of Isomer B was present in the tetrasaccharide acid fraction. Some Isomer A also appeared to have been present.

If it is assumed that all degradation, resulting in product loss, was non-selective, then the material isolated was representative of the purified aldotetraouronic acid fraction. It follows, therefore, that Isomer B was the predominant aldotetraouronic acid in the original fraction. This hypothesis is consistent with assumption (b), discussed above, which suggested that the di-O-methylxylose peaks, 4, 5, 6, and 7, in Fig. 14, were not caused entirely by these methylated components. The areas of these peaks thus may be partially attributed to impurities arising in the reaction sequence utilized for the structural investigation. The nature of the impurities is not known, but it seems likely that they were destroyed during gas chromatography, since very little material corresponding to these peaks could be isolated. It is difficult to imagine a substance to which the hydrogen flame detector would be so sensitive that practically no material could correspond to such an appreciable peak. Since this detector responds to the combustion products of each component, rather than to the component itself, the hydrogen flame detector might be expected to respond in the same manner to a given component and its thermal decomposition products.

An alternative explanation, based on incomplete methanolysis, may also be advanced to account for the apparently anomalous gas chromatographic data of $M_1$, Table IV. This hypothesis is not particularly satisfactory, however, for at least one very good reason. The distribution of methylated components noted in the gas chromatographic analysis data was not consistent with any conceivable partial methanolysis scheme. This was because any such scheme which yielded either 3,4-di-O-methylxylose, arising from Isomer A, or 3-O-methylxylose, arising from Isomer B, must give an equivalent amount of 2,3,4-tri-O-methylglucose. Thus, even in a case involving incomplete methanolysis, the moles of 3,4-di-O-methylxylose plus the moles of 3-O-methylxylose must equal the moles of 2,3,4-tri-O-methylglucose. As discussed previously, this was not the case; these three methylated components appeared, in the gas chromatographic analysis, to be present in a 1:1:1 molar ratio.

The two mild acid hydrolysis experiments, when considered together, were consistent only with Isomer B. Although this was not conclusive evidence, it did suggest that Isomer B was the predominant component of the purified aldotetraouronic acid. These observations, by supporting the predominance of Isomer B, also support the validity of assumption (b), i.e., the di-O-methylxylose peak areas in Fig. 14 were partially spurious. Any large quantity of Isomer A in the purified aldotetraouronic acid fraction would have altered the mild acid hydrolysis results.

On the basis of the foregoing discussion, it is suggested that Isomer B was the principal component, approximately 94 mole per cent, of the purified aldotetraouronic acid fraction. (It should be emphasized again that the available quantitative data are not conclusive on the per cent of each isomer.) From the information obtained in the structural investigation and also from the known

structure of the parent 4-O-methylglucuronoxylan, the specific name of Isomer B may be given to the principal component of the purified aldotetraouronic acid fraction: Isomer B: O-β-D-xylopyranosyl-(1⟶4)-O-[4-O-methyl-α-D-glucurono-pyranosyl-(1⟶2)]-O-β-D-xylopyranosyl-(1⟶4)-D-xylopyranose.

A second component, apparently Isomer A, is suggested to have been a minor component in the purified aldotetraouronic acid. Timell (34) also has isolated this linear tetrasaccharide acid, from the enzymatic hydrolysis of the same hemicellulose, and given its specific name: Isomer A: 4-O-methyl-α-D-glucurono-pyranosyl-(1⟶2)-O-β-D-xylopyranosyl-(1⟶4)-O-β-D-xylopyranosyl-(1⟶4)-D-xylopyranose.

## RELATIVE BOND STABILITIES IN THE 4-O-METHYLGLUCURONOXYLAN

The relative stability to acid hydrolysis of the five glycosidic linkages in the polymer segment illustrated in Fig. 17, may be deduced from the isomeric distribution in the tetrasaccharide acid fraction. (In this discussion, all bond designations, such as bond a or bond b, will refer to Fig. 17.) Four of these linkages, bonds a, b, c, and d, occur in various combinations in the three isomers. The fifth linkage, bond e, is common to all three isomers.

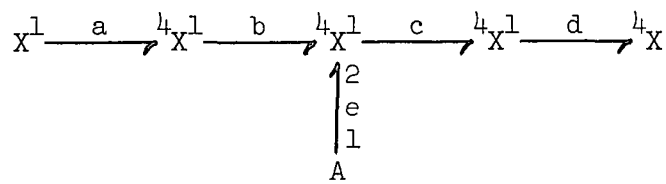



Figure 17. Segment of the 4-O-Methylglucuronoxylan

In the formation of any of the three aldotetraouronic acid isomers, there is a stability "competition" between three pairs of bonds in the hemicellulose polymer. The bond pairs involved in this competition are a and c, b and c, and a and d. As shown in Table V, cleavage in various combinations of each of these four bonds leads to Isomers A, B, and C. The quantity of each isomer should be an indirect measure of the relative stability of these four glycosidic linkages.

TABLE V

OCCURRENCE OF GLYCOSIDIC LINKAGES IN THE ALDO-TETRAOURONIC ACIDS IN FRACTION B

| Isomer | a | b | c | d | e | % Isolated |
|---|---|---|---|---|---|---|
| A | | | x | x | x | 6 |
| B | | x | x | | x | 94 |
| C | x | x | | | x | 0 |

As previously discussed, Isomer B was the most stable isomer, Isomer A the second most stable, and Isomer C the least stable. From the stability of these isomers, the outcome of the "competition" between bond pairs a and c, b and c, and a and d, may be deduced. From Table V, it may be seen that bond c must be more stable than either bond a, bond b, or bond d because bond c appears in both of the two isomers recovered, while the other three bonds do not. Bond b, in turn, must be more stable than either bond a or bond d, since bond b (appearing in Isomer B) occurs with greater frequency. Bond a, found only in Isomer C, did not survive because it was in direct competition with the extremely stable bond c. Bond a and bond d were assumed to be of similar stability, due to their separation from the branch point. These two bonds may well be typical xylose-xylose $\beta$-1,4 glycosidic linkages. On the basis of this discussion, a sequence of bond stabilities, with respect to acid hydrolysis, may be hypothesized: bond e > bond c > bond b > bond a $\simeq$ bond d.

The stability of bond c is consistent with the conformation stabilization hypothesis of McKee and Dickey (19). The bulky acid group substituted at $C_2$ of the xylose moiety restricts rotation about the $C_2$–$C_3$ carbon-carbon bond and the adoption of the half-chair hydrolysis intermediate is hindered. Extension of McKee's hypothesis to the aldotetraouronic acid is not entirely satisfactory, however, since the expected quantity of Isomer A was not obtained, although the quantitative data are not conclusive. This hypothesis did not anticipate stabilization of bond b and modification of this theory is required.

The Hirshfelder model of Isomer B was utilized to examine the steric factors which might stabilize bond b. The central xylose unit was allowed to adopt a flattened chair conformation, as suggested by the recent nuclear magnetic resonance studies of Lenz and Heeschen (40), van der Veen (41), Ferrier and Singleton (42), and Lemieux and co-workers (43). In this arrangement, the 4-O-methylglucuronic acid side chain becomes available for interactions with the glycosidic oxygen of bond b.

The exact nature of this interaction is unknown, but three possibilities may be suggested:

(1) The presence of the bulky acid side chain may sterically hinder protonation of bond b;

(2) The carboxyl group of the acid side chain, acting as a dipole, may repel the approach of protons through an electrostatic field effect;

(3) Hydrogen bonding, between the carboxyl proton and the ring oxygen of the nonreducing end xylose unit, may provide an energy barrier to the hydrolytic scission of bond b.

On the basis of the available data, it is not possible to evaluate effectively

these three postulated interactions. There may, in fact, be some combination of these factors which is responsible for the stability of bond b.

The extension of the theory of Marchessault and Rånby (11) to the aldotetraouronic acid does not appear to have been valid. Although the relative quantities isolated of Isomer A and Isomer B could not be definitely determined, a significant quantity of Isomer B was present. It is clear, therefore, that the "activation" of bond b, anticipated by Marchessault and Rånby did not occur. Therefore, the application of their theory to the hydrolysis of the xylose – xylose linkages in the 4-O-methylglucuronoxylan does not appear to be legitimate.

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## LITERATURE CITED


1. Shafizadeh, F., Adv. Carbohydrate Chem. 13:9-16(1958).
2. Capon, B., and Overend, W. G., Adv. Carbohydrate Chem. 15:11-51(1960).
3. McIntyre, D., and Long, F. A., J. Am. Chem. Soc. 76:3240-2(1954).
4. Bunton, C. A., Lewis, T. A., Llewellyn, D. R., and Vernon, C. A., J. Chem. Soc. 1955:4419-23.
5. Armour, C., Bunton, C. A., Patai, S., Selman, L. H., and Vernon, C. A., J. Chem. Soc. 1961:412-16.
6. Banks, B. E. C., Meinwald, Y., Rhind-Tutt, A. J., Sheft, I., and Vernon, C. A., J. Chem. Soc. 1961:3240-6.
7. Edward, J. T., Chem. and Ind. 1955:1102-4.
8. Reeves, R. E., J. Am. Chem. Soc. 71:215-17(1949).
9. Newman, M. S., J. Chem. Educ. 32:344-7(1955).
10. Dyer, E., Glaudemans, C. P. J., Koch, M. J., and Marchessault, R. H., J. Chem. Soc. 1962:3361-4.
11. Marchessault, R. H., and Rånby, B. G., Svensk Papperstid. 62:230-40(1959).
12. Whistler, R. L., and Richards, G. N., J. Am. Chem. Soc. 80:4888-91(1958).
13. Hamilton, J. K., and Thompson, N. S., Pulp Paper Mag. Can. 61:263-72(1960).
14. Morell, S., and Link, K. P., J. Biol. Chem. 104:183-8(1934).
15. Nakano, J., and Rånby, B. G., Svensk Papperstid. 65:29-33(1962).
16. Easty, D. B., J. Org. Chem. 27:2102-6(1962).
17. Semke, Leon K. The rates of acid hydrolysis of the beta-D-glucopyranosid-uronic acids and beta-D-glucopyranosides of phenol, para-cresol and para-chlorophenol. Doctor's Dissertation. Appleton, Wisconsin, The Institute of Paper Chemistry, 1963. 135 p.
18. Hamilton, J. K., and Thompson, N. S., J. Am. Chem. Soc. 79:6464-9(1957).
19. McKee, S. C., and Dickey, E. E., J. Org. Chem. 28:1561-3(1962).
20. Whistler, R. L., and Durso, D. F., J. Am. Chem. Soc. 74:5150-1(1952).
21. Overend, W. G., Rees, C. W., and Sequeira, J. S., J. Chem. Soc. 1962:3429-40.

22. Timell, T. E., Chem. and Ind. 1963:1208-9.

23. Anderson, E., and Sand, L., Adv. Carbohydrate Chem. 1:329-44(1945).

24. Jones, J. K. N., and Smith, F., Adv. Carbohydrate Chem. 4:243-91(1949).

25. Whistler, R. L., Adv. Carbohydrate Chem. 5:269-90(1950).

26. Polglase, W. J., Adv. Carbohydrate Chem. 10:283-333(1955).

27. Aspinall, G. O., Adv. Carbohydrate Chem. 14:429-68(1959).

28. Timell, T. E., Tappi 40:568-72(1957).

29. Glaudemans, C. P. J., and Timell, T. E., J. Am. Chem. Soc. 80:941-3(1958).

30. Glaudemans, C. P. J., and Timell, T. E., J. Am. Chem. Soc. 80:1209-13(1958).

31. Timell, T. E., Svensk Papperstid. 65:435-47(1962).

32. Gorin, P. A. J., and Perlin, A. S., Can. J. Chem. 36:999-1003(1958).

33. Johansson, I., Lindberg, B., and Theander, O., Acta Chem. Scand. 17:2019-24 (1963).

34. Timell, T. E., Can. J. Chem. 40:22-5(1962).

35. McDonald, I. R. C., J. Chem. Soc. 1952:3183-6.

36. Hamilton, J. K., and Thompson, N. S., Tappi 42:752-60(1959).

37. Bishop, C. T., Meth. Biochem. Anal. 10:1-42(1962).

38. Bishop, C. T., Can. J. Chem. 34:1255-60(1956).

39. Srivastava, H. C., Bishop, C. T., and Adams, G. A., J. Org. Chem. 26:3958-60 (1961).

40. Lenz, R. W., and Heeschen, J. P., J. Polymer Sci. 51:247-61(1962).

41. van der Veen, J. M., J. Org. Chem. 28:564-6(1963).

42. Ferrier, R. J., and Singleton, M. F., Tetrahedron 18:1143-8(1962).

43. Lemieux, R. U., Kullnig, R. K., Bernstein, H. J., and Schneider, W. G., J. Am. Chem. Soc. 80:6098-105(1958).

44. Brasch, D. J., and Wise, L. E., Tappi 39:581-8(1956).

45. Dickey, E. E. Laboratory experiments in the chemistry of wood. Appleton, Wis., The Institute of Paper Chemistry, 1962. 46 p.

46. Thompson, N. S. Personal communication, 1963.

47. Hamilton, J. K., and Thompson, N. S., Tappi 42:752-60(1959).

48. Green, J. W. Personal communication, 1964.

49. Adams, G. A., Can. J. Chem. 30:698-710(1952).

50. Bishop, C. T., J. Am. Chem. Soc. 78:2840-1(1956); Bishop, C. T., Can. J. Chem. 31:134-44(1953).

51. Aspinall, G. O., and Mahomed, R. S., J. Chem. Soc. 1954:1731-4.

52. Roudier, A., and Bertrand, G., Compt. Rend. 248:1432-5(1959).

53. Aspinall, G. O., and Meek, E. G., J. Chem. Soc. 1954:3830-4.

# APPENDIX I

## PAPER CHROMATOGRAPHY

### SOLVENTS AND SPRAYS

Solvent A: 18:3:1:4 (v/v) Ethyl acetate:acetic acid:formic acid:water (44)

Solvent B: 6:3:2 (v/v) Ethyl acetate:acetic acid:water (18)

Solvent C: 8:2:1 (v/v) Ethyl acetate:pyridine:water (45)

Solvent D: 2-Butanone:water azeotrope (18)

Spray A: p-Anisidine hydrochloride (0.5 g.), distilled water (5 ml.), absolute ethanol (10 ml.), n-butanol (85 ml.). Spray and dry sheet, then heat for five minutes at 105°C. to develop the spots (45).

Spray B: Add trichloroacetic acid (3.0 g.) to Spray A. Treat in the same manner (46).

Spray C: (1) Silver nitrate (3.0 g.), distilled water (5 ml.), and acetone (95 ml.). (2) Sodium hydroxide (2 g.), distilled water (5 ml.), and absolute ethanol (95 ml.). (3) Sodium thiosulfate (10 g.), and distilled water (90 ml.). The sheets were dipped and dried successively in the three solutions described (45).

### PREPARATIVE PAPER CHROMATOGRAPHY (46, 47)

Sheets of Whatman No. 17 paper (18-1/2 by 22-1/2 inches) were prepared with a support loop and wick of Whatman no. 1 paper, as shown in Fig. 18. The oligouronide sample, containing approximately 100 mg. of solids (dissolved in a 50% ethanol solution) per linear inch of starting line, was placed in a thin strip on the chromatogram. The starting line was located about 1/2 inch below the support loop. The sheet was irrigated with the appropriate solvent in the usual manner for 72 hours. After drying, three guide strips for each sheet were prepared by applying, with considerable pressure, 3/4-inch strips of cellophane tape from the starting line to the bottom edge of the sheet. The tape strips

were "zipped" off and the thin fiber layer treated with Spray A. The several fractions were easily located with minimum material loss (48). Similar fractions were combined and eluted with distilled water, by means of the elutions packets shown in Fig. 19. Overnight elution was sufficient to remove all reducing material from the sheets.

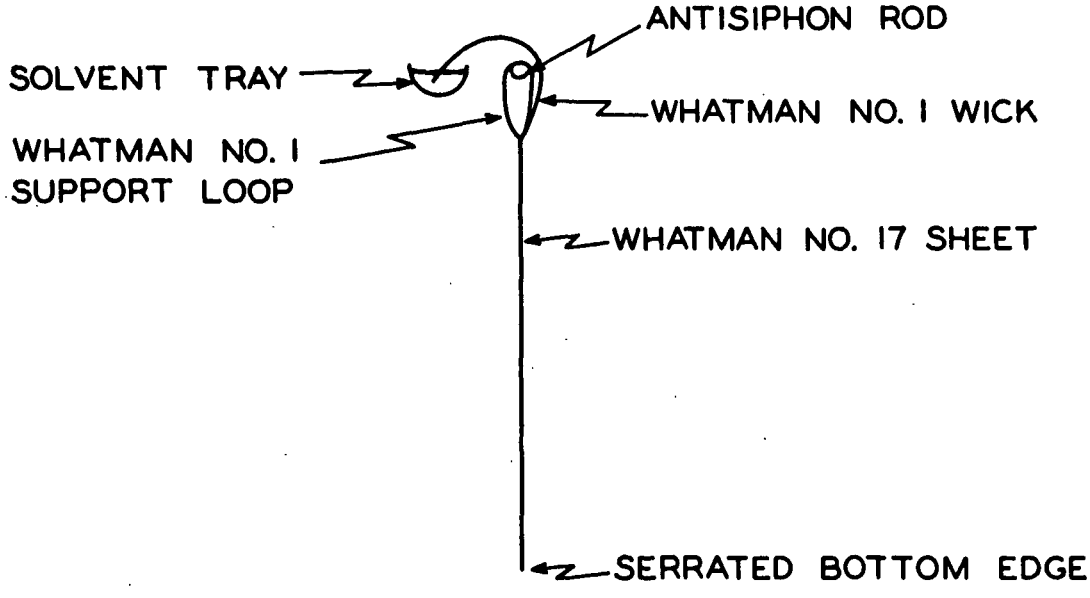



Figure 18. Heavy Paper Chromatograms (Side View)

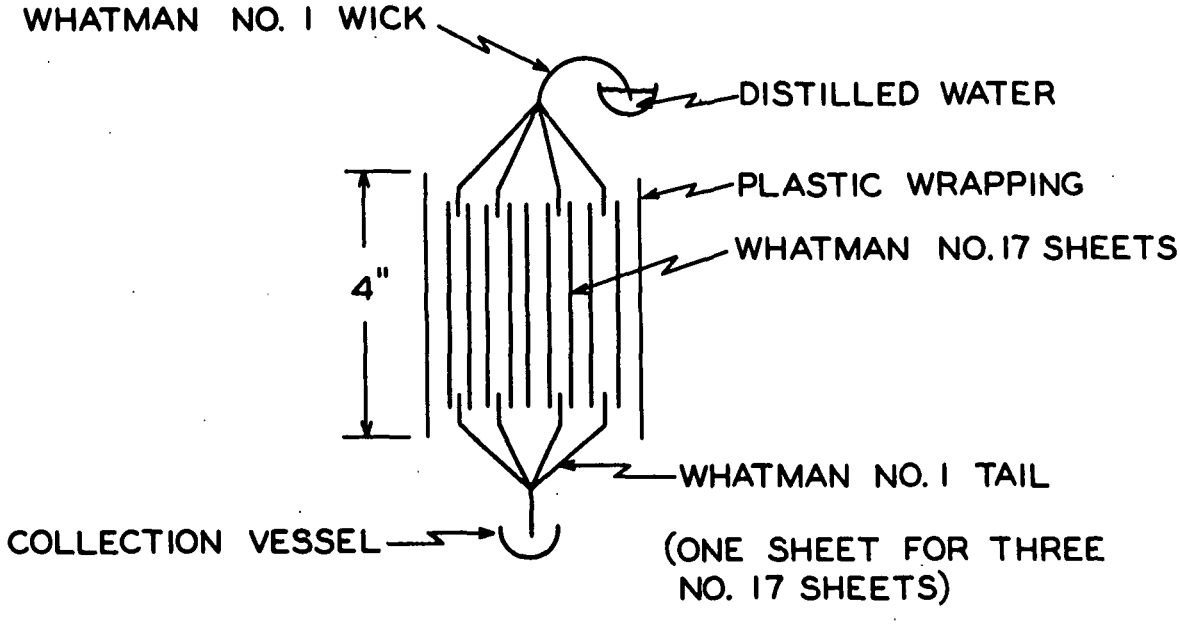



Figure 19. Elution Packets (Side View)

# APPENDIX II

## ELECTROPHORESIS

### QUALITATIVE PAPER ELECTROPHORESIS

Sheets of Whatman No. 1 paper were prepared in strips 7 by 23 inches. A 40-60 microgram sample of each sugar was applied in a single spot on a line approximately nine inches from one end of the paper. No more than five sugar samples were examined at one time. The sheet was saturated with 0.1M sodium borate solution and placed between two glass sheets in the electrophoresis apparatus (Research Equipment Corporation, Oakland, California Model E-800-2). A schematic sketch of this apparatus is shown in Fig. 20. The short end of the paper strip was placed toward the cathode and the ends of the strip dipped in 0.1M sodium borate solution. A potential of 500 volts was applied for three hours. The sheets were removed after completion of the electrophoresis, air dried, and treated with chromatographic Spray B to locate the various sugars.

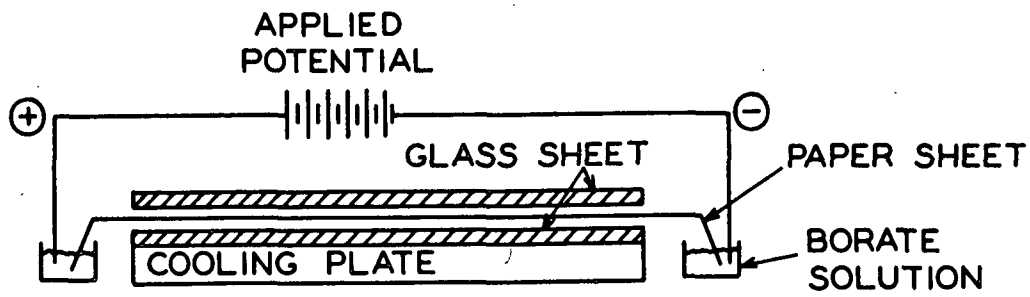



Figure 20. Electrophoresis Apparatus

## PREPARATIVE ELECTROPHORESIS

Sheets of Whatman No. 17 paper were prepared similar to those used for qualitative electrophoresis. A sample of 400-600 mg. was placed on the starting line, the sheet saturated with 0.1M sodium borate, and the sheet placed in the apparatus, as previously described. A potential of 360 volts was applied for five hours. Before removing the sheet from the electrophoresis apparatus, a dry Whatman No. 1 sheet was carefully superimposed on the preparative electrophoretogram. As soon as the No. 1 sheet was saturated, both sheets were removed from the apparatus and air dried. The Whatman No. 1 sheet was then treated with Spray B to locate the various fractions. These fractions were then isolated in the usual manner, described in Appendix I.

# APPENDIX III

## INFRARED SPECTRAL CRITERION FOR COMPLETE METHYLATION

In order to evaluate the validity of this technique, several known samples were investigated. In no case was complete removal of the hydroxyl stretching band, at 2.85 microns, attained. It is suspected that residual moisture was responsible for these minor peaks, amounting to no more than five percentage transmittance points. The value for the aldotetraouronic acid was two percentage points.

1. 2,3,4-tri-O-methyl β-methyl xylopyranoside: This crystalline material, melting point 48.5-49.5°C. (corr.), was isolated by gas chromatography from the methylation of xylose. The sample was considered to be extremely pure. The IR spectra showed a minor peak at 2.85 microns, when run in chloroform using the dual beam feature of the spectrometer.

2. The fully methylated, nonreducing trisaccharide acid. Experimental % methoxyl: 53; theoretical: 52.7. The IR spectra showed a small peak at 2.85 microns when run between NaCl plates as the dried sirup.

3. The fully methylated, nonreducing tetrasaccharide acid. Experimental % methoxyl: 46; theoretical: 49.7. The IR spectra showed a small peak at 2.85 microns when run between NaCl plates.

# APPENDIX IV

## GAS CHROMATOGRAPHY

A gas chromatography column was obtained (from the Wilkins Instrument Company, Walnut Creek, California) for the Aerograph Hi Fi Gas chromatograph, Model A 600-B. This four-foot column was packed with 80/100 mesh, acid-washed Chromosorb P (a pink diatomaceous earth) coated with 15% butane-1,4-diol succinate polyester. The column was scrubbed for several days at 200°C. with 24 ml./min. of prepurified nitrogen gas. The background reading, due to continuing loss of column liquid phase, varied but remained below 5 divisions (peak height) at 1x (attenuation) and $10^9$ (input impedance) at 175°C. (column temperature).

All samples were run at $10^7$ input impedance and from 1x to 8x attenuation. Samples of from 1 to 10 microliters were usually found to be satisfactory. The sample was injected into the sample port, as shown in the schematic representation in Fig. 21. The sample was transported and fractionated through the column by the carrier gas. The various components were located, in the carrier gas stream, by the hydrogen flame detector. As each component emerged from the column, it was burned in the hydrogen flame. Changes in the composition of the hydrogen flame were noted by the electrometer circuit. The electrometer output was recorded on a strip recorder. All quantitative and qualitative work was done on the Model A 600-B.

Gas chromatographic isolations were carried out on the Aerograph A-90s. This instrument is arranged in a manner similar to the A 600-B. In place of the destructive hydrogen flame detector, the A-90s utilizes a thermal conductivity detector. This detector is nondestructive, with respect to the sample, and permits sample collection. A U tube was constructed which could be attached

to the exit port of the A-90s. As illustrated in Fig. 22, the bend of the collecting tube was immersed in a dry ice-acetone bath. A glass wool plug was placed in the exit leg of the collecting tube to insure collection of "aerosol" samples. The samples were removed from the collecting tubes by washing with chloroform.

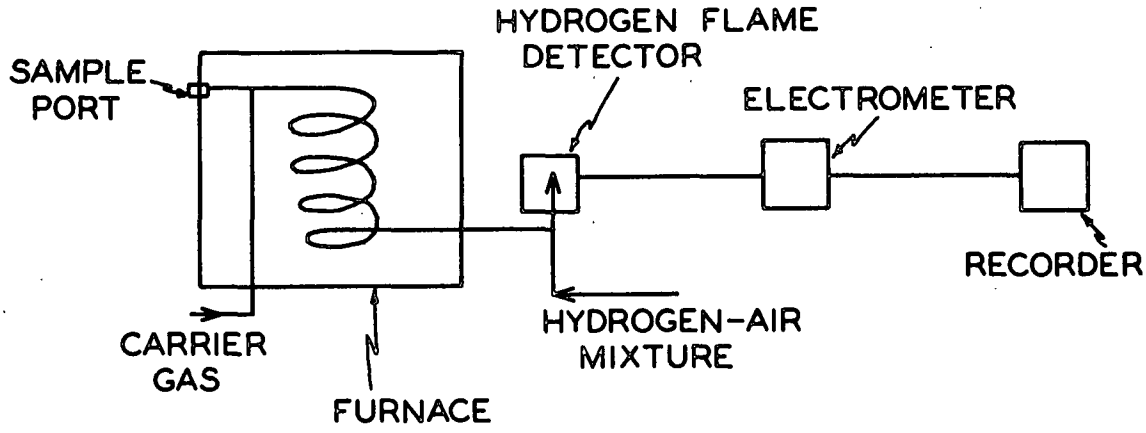



Figure 21. Schematic Representation of the A 600-B

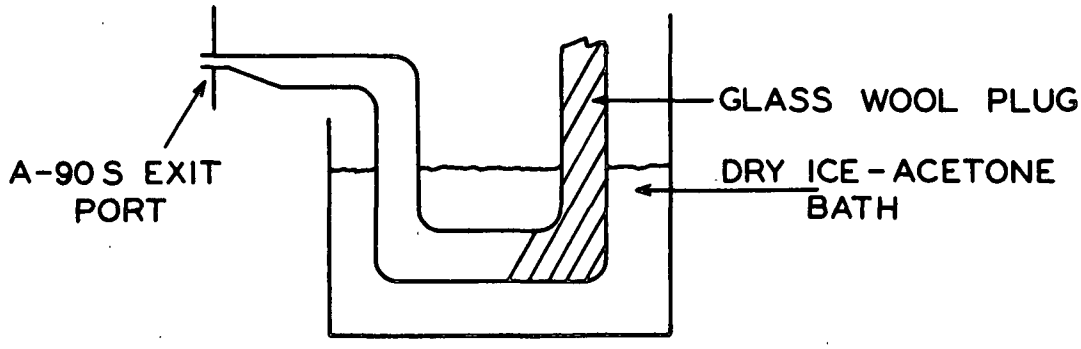



Figure 22. Gas Chromatography Collection Tube

The response of the A 600-B Gas Chromatograph was calibrated by examination of three solutions of known composition: (1) Methyl 2,3,4-tri-O-methyl-α,β-D-xylopyranoside and methyl 3-O-methyl-α,β-D-xylopyranoside, in a 1.1 to

1.0 molar ratio were prepared by methanolysis of the crystalline sugars. (2) Xylobiose, 50 mg. identified by its paper chromatographic mobility (in Solvent A, $R_X$ = 0.53; in Solvent C, $R_X$ = 0.51) and hydrolysis product (xylose, $R_X$ = 1.00 in Solvents A and C), was reduced with sodium borohydride and methylated with silver oxide – methyl iodide, as previously described for the aldotetraouronic acid. Methanolysis of the product yielded a mixture of methyl 2,3,4-tri-O-methyl-α,β-D-xylopyranoside and 1,2,3,5-tetra-O-methylxylitol in a 1.0 to 1.0 molar ratio. (3) Authentic crystalline aldotriouronic acid, 50 mg., was treated in the same manner as the aldotetraouronic acid (see p. 21-2), i.e., sodium borohydride reduction, methylation, and lithium aluminum hydride reduction. Methanolysis of the product (the methylated, nonreducing neutral trisaccharide) yielded a mixture of methyl 2,3,4-tri-O-methyl-α,β-D-glucopyranoside, 1,2,3,5-tetra-O-methylxylitol, and methyl 3,4-di-O-methyl-α,β-D-xylopyranoside in a 1.0:1.0:1.0 molar ratio. The area of the dimethylxylose peaks could not be determined because of excessive peak overlap and distortion.

The area of each peak was measured and a factor calculated to make the peak areas proportional to the molar ratios. The peak area factor for 2,3,4-tri-O-methylxylose was set equal to 1.00 arbitrarily. The data appear in Table VI.

TABLE VI

CALIBRATION OF A 600-B RESPONSE

| Solution | Component | Molar Ratio | Peak Area | Factor |
|---|---|---|---|---|
| 1 | Trimethylxylose | 1.1:1.0 | 62.0 | 1.00 |
| | Monomethylxylose | | 47.3 | 1.44 |
| 2 | Trimethylxylose | 1.0:1.0 | 39.1 | 1.00 |
| | Tetramethylxylitol | | 33.8 | 1.16 |
| 3 | Tetramethylxylitol | 1.0:1.0 | 57.4 | 1.16 |
| | Trimethylglucose | | 70.8 | 0.95 |

# APPENDIX V

## STUDIES ON WHEAT STRAW

### 4-O-METHYLGLUCURONOXYLAN

The exact nature of the linkage between the 4-O-methylglucuronic acid branch and the xylose polymer backbone has not been resolved. Adams (49), Bishop (50), and Aspinall and Mahomed (51) have suggested an α-1,3 linkage. Roudier and Bertrand (52), and Aspinall and Meek (53) have supported the usual α-1,2 linkage. The β-1,4 linkage of the xylose units in the polymer backbone is commonly accepted by these workers.

In this work, a holocellulose was prepared by IPC Method 28 (at 70°C.) from 3.6 kg. of wheat straw meal (Selkirk spring wheat variety); yield: 2.7 kg. (o.d.). The undried holocellulose was extracted on a table top Buchner funnel with 30 ℓ of 4% KOH. The extract was removed continuously for four hours. The alkaline extract was neutralized to pH 5 with acetic acid and then added to two volumes of 95% ethanol. The precipitate was allowed to settle and the supernatant decanted and discarded. A second volume of ethanol was added and, after standing overnight, the precipitated hemicellulose was isolated by filtration; yield: 660 g. (o.d.).

The glucuronoxylan (600 g.) was dispersed in N sulfuric acid (10.8 ℓ.) and stirred for 10 hours at 70°C. Following cooling, the reaction mixture was neutralized to pH 5 with solid barium carbonate. The solids were removed by centrifugation and the filtrate concentrated to a heavy sirup; yield: 62.5 g. (o.d.).

The hydrolyzate sirup (60 g.) was evaporated to dryness, under reduced pressure, in the presence of solid barium carbonate. The residue was divided into three equal parts and each of these dispersed in 500 ml. of absolute ethanol. The three samples were shaken continuously for two weeks, with occasional changes in ethanol. The powdery product was dissolved in distilled water and deionized on IR-120 ($H^+$). The oligouronide mixture was then separated into six fractions by heavy paper chromatography.

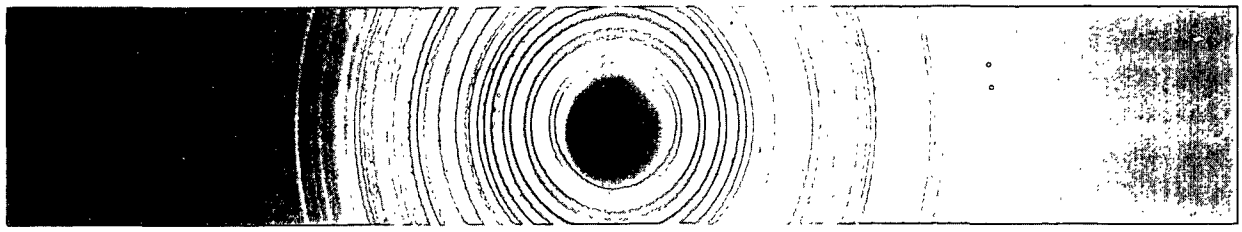
One of these fractions, on further purification, yielded 1.6 grams of a crystalline aldotriouronic acid. As shown by the data summarized in Table VII this aldotriouronic acid was identical to the common trisaccharide acid (18).

TABLE VII

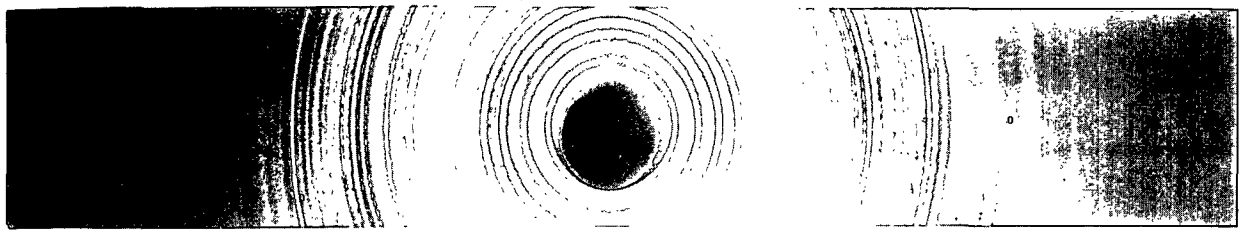
COMPARISON OF KNOWN AND SUSPECTED ALDOTRIOURONIC ACIDS

| Characteristic of $\underline{X}_2\underline{A}$ | Known Acid | Suspected Acid |
|---|---|---|
| Melting point | 184°C. (18) | 184-186°C. |
| $R_{\underline{X}}$ in Solvent A | 0.43 | 0.43 |
| Hydrolysis products | $\underline{X}$, $\underline{A}$ | $\underline{X}$, $\underline{A}$ |
| Infrared spectra | Identical | |
| X-ray diffraction pattern | Identical (see Fig. 23) | |
| Equivalent weight (potentiometric titration) | 526 | 504 |

No evidence of a second and different trisaccharide acid fraction could be found. It was, therefore, concluded that the linkage between the acidic side chain and the xylan polymer backbone was the common $\alpha$-1,2 glycosidic bond.



KNOWN TRISACCHARIDE ACID (18)



SUSPECTED TRISACCHARIDE ACID

Figure 23. X-ray Diffractograms of Known and Suspected Aldotriouronic Acids

## AN ALDOTETRAOURONIC ACID

A second fraction, from the acid hydrolyzate of the wheat straw hemicellulose, yielded an aldotetraouronic acid. This acid was apparently quite similar to Fraction (II), isolated from the hydrolyzate of the paper birch hemicellulose. Pertinent data supporting this contention appear in Table VIII.

TABLE VIII

COMPARISON OF TWO ALDOTETRAOURONIC ACIDS

| Characteristic | Wheat Straw | Paper Birch |
|---|---|---|
| Chromatographic mobility, $R_{\underline{X}}$ in Solvent A | 0.14 | 0.14 |
| Equivalent weight by potentiometric titration | 604 ± 5% | 604 ± 3% |
| X:A ratio $LiAlH_4$ reduction/hydrolysis | 3:1 | 3:1 |
| Final hydrolysis products | X, A | X, A |
| Mild acid hydrolysis (0.5N $H_2SO_4$/70°C.) | X, $X_2A$ | X, $X_2A$ |
| $NaBH_4$ reduction/mild acid hydrolysis | X, $X_2A$* | X, $X_2A$* |

(X = xylose; A = 4-O-methylglucuronic acid; $X_2A$ = common aldotriouronic acid; $X_2A$* = nonreducing analog of the common aldotriouronic acid.)

## APPENDIX VI

## GLOSSARY

$\underline{X}$ = xylopyranose, $R_{\underline{x}}$ = 1.00 in Solvents A, B, and C

$\underline{X}^*$ = xylitol, $R_{\underline{x}}$ = 1.10 in Solvent A; $R_{\underline{x}}$ = 0.66 in Solvent B

$\underline{A}$ = 4-O-methylglucuronic acid

$\underline{X}_2\underline{A}$ = common aldotriouronic acid, $R_{\underline{x}}$ = 0.45 in Solvent A

$\underline{X}_3\underline{A}$ = aldotetraouronic acid, $R_{\underline{x}}$ = 0.14 in Solvent A

M = mannopyranose

gal = galactopyranose

R = aglycon

$C_{\underline{n}}$ = carbon atom, where $\underline{n}$ is an integer denoting location in the pyranose ring

O = oxygen atom

H = hydrogen atom

$R_{\underline{x}}$ = distance traveled in cm. of a particular sugar divided by the distance in cm. traveled by xylose in the same solvent and in the same time (paper chromatography)

$M_{\underline{g}}$ = distance traveled in cm. of a particular sugar divided by the distance traveled in cm. of glucose, under the same conditions (electrophoresis)

Fractions (I), (II), and (III) = three fractions isolated on electrophoresis of the crude aldotetraouronic acid (see p. 20)

Isomers A, B, C = the three theoretically possible aldotetraouronic acid isomers (see p. 16)

Bonds a, b, c, d, and e = glycosidic linkages in 4-O-methylglucuronoxylan (see p. 34)

o.d. = ovendry, overnight at 105°C. Moisture determinations were generally carried out on an aliquot

$M_{\underline{1}}$ = mole ratio calculated from gas chromatographic data, $R_{\underline{r}}$

$M_{\underline{2}}$ = mole ratio calculated from isolation data, $\underline{F}$

$T_{\underline{r}}$ = gas chromatographic retention time

$\underline{A_r}$ = gas chromatographic peak area in square units

$\underline{C_f}$ = calibration factor for converting gas chromatographic peak areas to the same basis, due to differences in instrument response

$\underline{I}$ = isolated fraction number from gas chromatographic isolation

$\underline{F}$ = isolated fraction weight in mg., of $\underline{I}$