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Doctor's Dissertation

An Investigation of the Hydrolysis of a Reduced 4-*O*-Methylglucuronoxylan

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AN INVESTIGATION OF THE HYDROLYSIS OF A REDUCED 4-O-METHYLGLUCURONOXYLAN

A thesis submitted by

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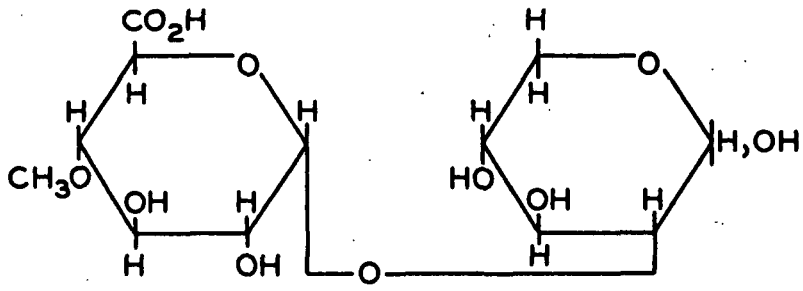
INTRODUCTION

4-O-METHYLGLUCURONOXYLAN

It is well known that most hardwoods contain 20-30% hemicellulose and the softwoods 15-20% hemicellulose. In the hardwoods, a 4-O-methylglucuronoxylan polymer predominates, while in the softwoods a glucomannan polymer is the major constituent. Other polymeric hemicellulose components of softwoods are galactoglucomannans and 4-O-methylglucuronoaraboxylan, while the hardwoods generally contain some glucomannan (1).

Structural chemistry of 4-O-methylglucuronoxylan has been reviewed by others (2, 3), so only the salient features will be cited. Early work (4) indicated that a methyl ether of a hexuronic acid was associated with the xylan of wood. D-xylose oligosaccharides and an aldobiouronic acid, 2-O-(4-O-methyl-α-D-glucopyranosyluronic acid)-D-xylopyranose (I) (Fig. 1), were isolated from a graded acid hydrolyzate of aspenwood (5).

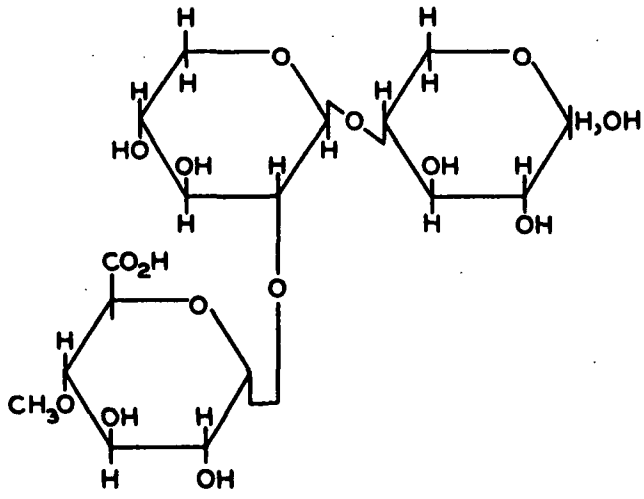


I

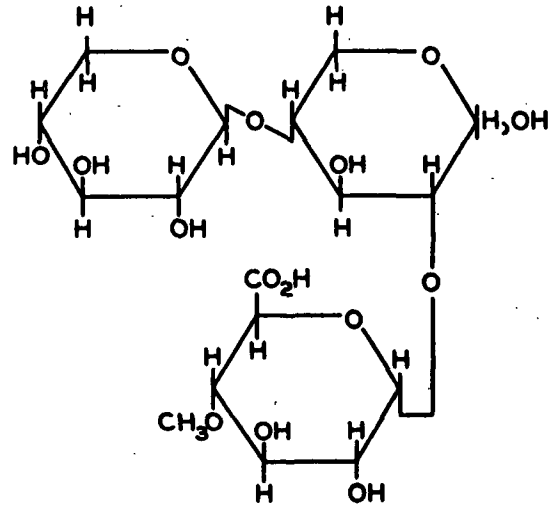
Figure 1. Aldobiouronic Acid [2-O-(4-O-methyl-α-D-glucopyranosyluronic acid)-D-xylopyranose]

The α-configuration of the glycosidic linkage in the aldobiouronic acid was established by the identity of a hexamethyl ether of 2-O-α-D-glucopyranosyl glycerol prepared from the aldobiouronic acid and from a known compound (6).

A crystalline aldotriouronic acid, O-α-4-O-methylglucuronopyranosyl (1→2)-O-β-D-xylopyranosyl-(1→4)-D-xylopyranose (II) (Fig. 2), was isolated and characterized (7). Final characterization of the aldotriouronic acid was based on the isolation of 2,3,4,6-tetra-O-methyl-D-glucose, 3,4-di-O-methyl-D-xylose and 1,2,3,5-tetra-O-methyl-D-xylitol after reduction of the methyl ester of (II) followed by methylation and hydrolysis (8, 9). Since aldotriouronic acid (II) has been isolated from aspen (10, 11), Monterey pine (12), Western hemlock (7), American elm (13), white spruce (14), and jute (8, 9), it can reasonably be called the ubiquitous aldotriouronic acid.



II
UBIQUITOUS ALDOTRIOURONIC ACID



III
("ISOMERIC" ALDOTRIOURONIC ACID)

Figure 2. Trisaccharide Acids

The isomer (III) (Fig. 2) of the ubiquitous aldotriouronic acid has never been obtained although considerable effort was expended in searching for it (7). Methylation and hydrolysis of an impure aldobiouronic acid yielded a trace of 2,3,4-tri-O-methyl-D-xylose (15). This finding was interpreted as indicating the possible presence of an aldotriouronic acid with structure (III). However, no real evidence for the presence of the isomeric aldotriouronic acid (III) has yet been presented.

The structure of a 4-O-methylglucuronoxylan from European beech was shown by methylation studies to consist of chains of 1→4 linked β-D-xylopyranose residues having single 4-O-methyl-D-glucuronic acid groups attached as side chains on C_2 of the xylose units (15). This conclusion was based primarily on the isolation of a large quantity of 2,3-di-O-methyl-D-xylose and an aldobiouronic acid with an unsubstituted C_4 position in the xylose moiety. The 4-O-methyl-glucuronoxylan structure is shown in Fig. 3.

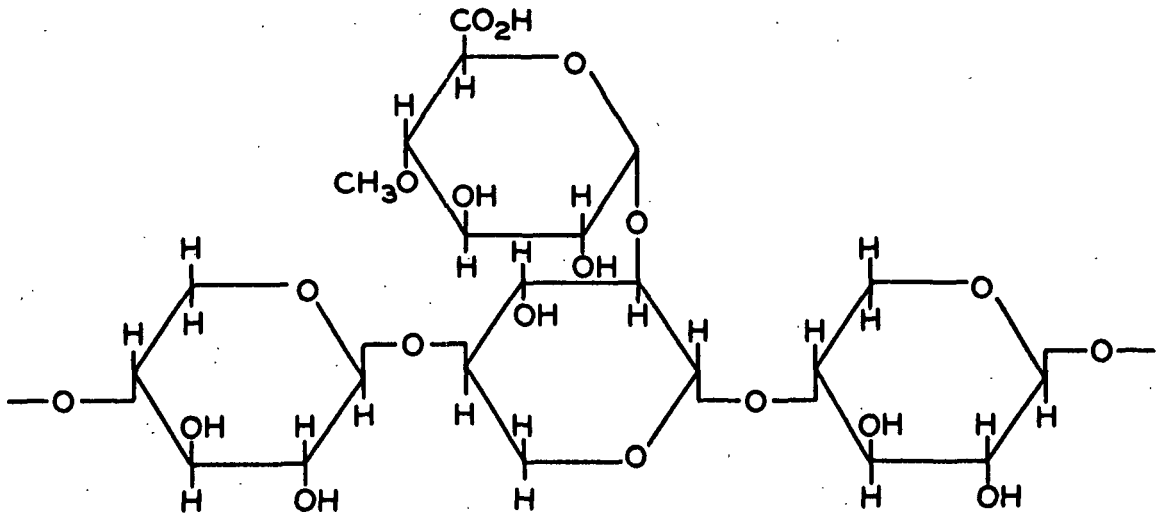


Figure 3. 4-O-Methylglucuronoxylan

Methylation studies on 4-O-methylglucuronoxylans from other hardwoods indicated the same general structure (3, 16). The isolation of monomethylxylose residues (13, 15, 16) could indicate branches of aldobiouronic acid or aldotriouronic acid. However, the unsubstituted C_4 position of the xylose moiety from aldobiouronic acid supports only the structure shown above. These monomethylxylose products may be the results of incomplete methylation or of demethylation (17, 18). Table I gives methylation data for several hardwood 4-O-methylglucuronoxylans.

TABLE I

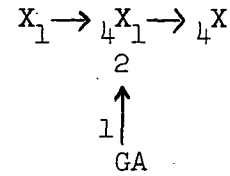
METHYLATED 4-O-METHYLGLUCURONOXYLANS FROM HARDWOODS

Molecular Properties	European Beech (15)	White Birch (17)	American Elm (13)
D.P. by 2,3,4-tri-O-methylxylose end group assay	75	124	145
D.P. by osmotic pressure or isothermal distillation	69	102	133
Methanolysis Products-Molar Ratios of Sugars			
2-O and 3-O-methyl-D-xylose	7	3	6
2,3-di-O-methyl-D-xylose	60	109	124
2,3,4-tri-O-methyl-D-xylose	1	1	1
Methyl 2-O-(2,3,4-tri-O-methyl-α-D-glucuronosyl)-3-O-methyl-D-xylopyranoside	7	11	14

Additional evidence favoring the linear xylan chain with branches of single 4-O-methylglucuronic acid units is obtained by comparison of the number average degree of polymerization (by osmotic pressure or isothermal distillation) with the degree of polymerization by 2,3,4-tri-O-methyl-D-xylose end-group assay (Table I). A branched molecule with a

single branch in the xylan chain would give a degree of polymerization by end-group assay of one-half the degree of polymerization by osmotic pressure. Thus, the data in Table I would indicate that the xylan chains are not branched.

Further confirmation of the accepted structure was supplied by an enzymatic, graded hydrolysis of white birch 4-O-methylglucuronoxylan which gave an aldotetrauronic acid with the following possible but unproven structure (19):



where X = β-D-xylopyranose unit
GA = 4-O-methyl-α-D-glucuronopyranose unit

A similar aldotetrauronic acid had been isolated earlier by enzymatic hydrolysis of hemlock polyuronide (20).

In its native state, the 4-O-methylglucuronoxylan exists as a partially acetylated polymer (21) with the acetyl groups located mainly on C_3 of the xylopyranose units (22, 23). Alkaline extraction commonly used for isolation of the 4-O-methylglucuronoxylan saponifies the acetyl groups with the result that 4-O-methylglucuronoxylan, as normally isolated, is free of acetyl groups but is assumed to be otherwise unchanged.

ACID-CATALYZED HYDROLYSIS OF GLYCOSIDES

MECHANISM OF HYDROLYSIS

Experiments on the acid-catalyzed hydrolysis of methyl and phenyl glucopyranosides indicated that the reaction was unimolecular with respect to the decomposition of the protonated glycoside, since a linear relationship was found between the first-order rate constant and the Hammett acidity function (24). The use of H_2O^{18} enriched solutions revealed that the reaction proceeded with fission of the hexose-oxygen bond (24), and two mechanisms were postulated to fit the experimental data (Fig. 4).

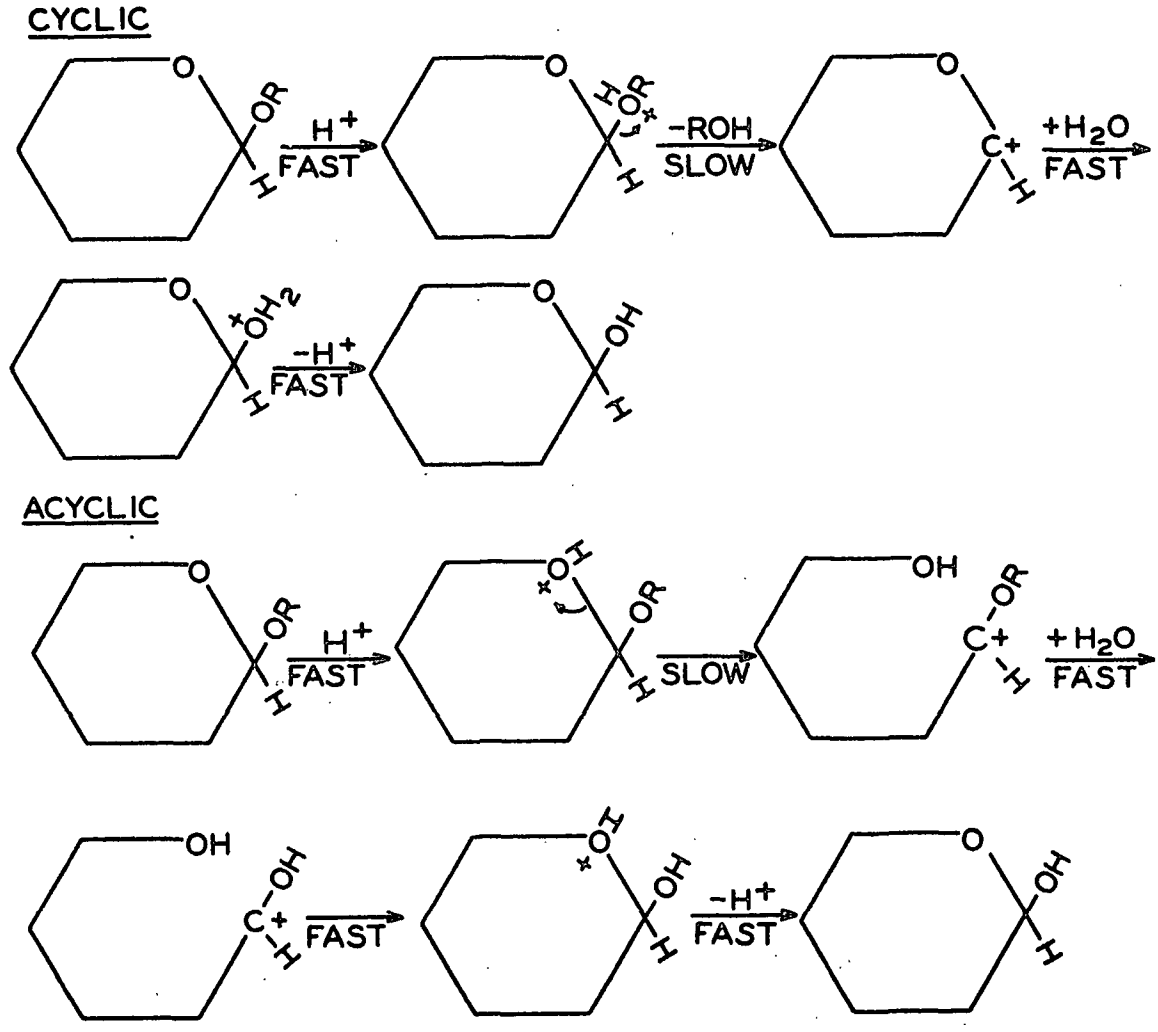


Figure 4. Mechanism for Acid-Catalyzed Hydrolysis of Methyl and Phenyl Glucopyranosides

FACTORS INFLUENCING HYDROLYSIS OF GLYCOSIDES

Since the factors influencing the acid-catalyzed hydrolysis of glycosides have been presented in detail by Shafizadeh (25), they will only be reviewed briefly here.

Generally, the steric strain of five and seven-membered rings causes an increase in rate of hydrolysis as compared with the less strained six-membered rings. The steric strain of six-membered rings in their favored chair conformation results from the nonbonded interaction of the axial substituents. Both α-D-glucopyranose and β-D-xylopyranose will be most stable in the C_1 conformation (26). As shown in Fig. 5, β-D-xylopyranose has all hydroxyl groups equatorial whereas α-D-glucopyranose has the glycosidic hydroxyl in an axial position.

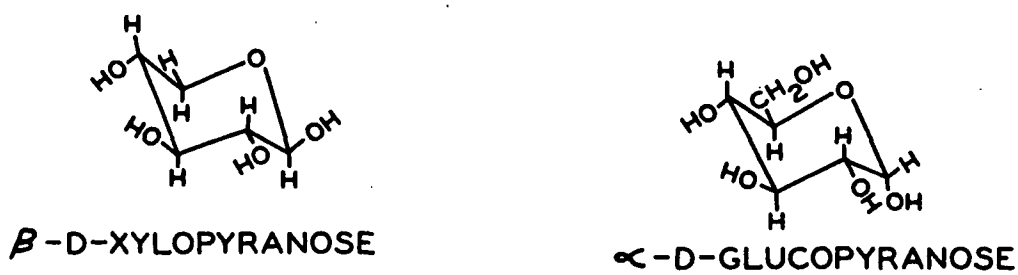


Figure 5. Conformations of β-D-xylopyranose and α-D-glucopyranose

The relative hydrolysis rates of some methylpyranosides are given in Table II. The case of hydrolysis of the α-anomer has been attributed to the easy protonization of the exposed equatorial group (25).

The nature of the aglycon has a considerable effect on the rate of glycoside hydrolysis. Greater stability of the α-anomer for methyl-glucopyranosides does not apply to disaccharides (cf. Table III).

TABLE II

HYDROLYSIS OF METHYLPYRANOSIDES IN 0.5N HCl AT 75°C. (25)

Aldose	Relative Rate Constant
α-D-glucose	1.0
β-D-glucose	1.9
α-D-xylose	4.5
β-D-xylose	9.0

The more rapid hydrolysis of maltose compared with cellobiose is probably due to instability resulting from the presence of large axial substituents (25).

TABLE III

HYDROLYSIS OF GLUCOPYRANOSIDES AT 60°C. (25)

	10^6 K, $sec.^{-1}$	ΔE, cal./mole
Methyl-α-D-glucopyranoside	1.46	38,190
Methyl-β-D-glucopyranoside	3.86	33,730
4-O-α-D-glucopyranosyl-D-glucopyranose (maltose)	16.80	30,970
4-O-β-D-glucopyranosyl-D-glucopyranose (cellobiose)	5.89	30,710

The substituents in the glycosyl unit can influence acid lability by three main effects: strain and conformational, masking of the reactive center, and polar effects (25).

On the assumption that the cyclic hydrolysis mechanism prevails, several explanations of observed hydrolysis rates have stressed the

importance of conformational aspects of the cyclic intermediates. It has been suggested that formation of the carbonium ion will cause the formation of a half-chair form (27-29) similar to cylohexene (30). The formation of the half-chair form for β-D-glucopyranose is illustrated in Fig. 6.

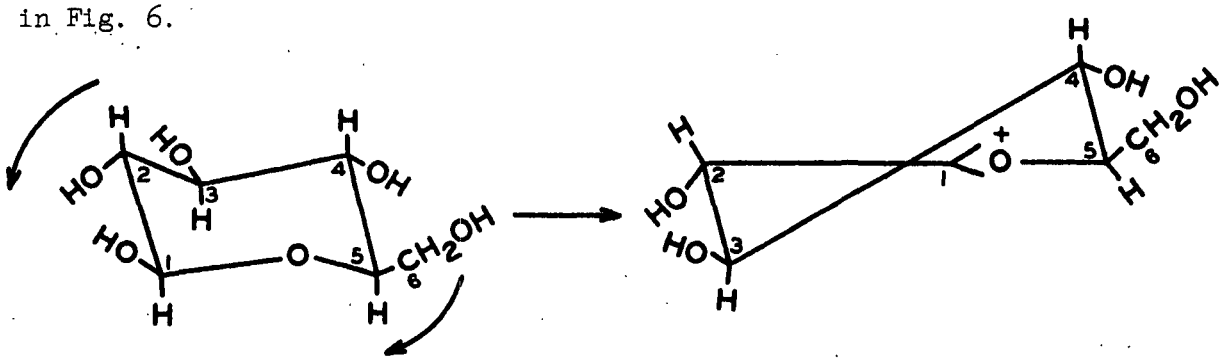
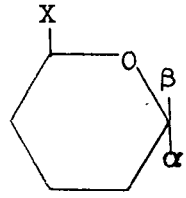


Figure 6. Formation of the Half-Chair Form for β-D-glucopyranose

The transformation from the puckered chair form to the planar half-chair form requires a small amount of rotation about the C_2-C_3 and C_4-C_5 bonds. The resistance to conform to the half-chair form is expected to increase with increasing size of substituents. The rates of glycoside hydrolysis for pyranosides with C_5 substituents of increasing size have been cited as evidence supporting this hypothesis (27, 28) (see Table IV).

TABLE IV

RELATIVE EASE OF HYDROLYSIS OF METHYL PENTOSIDES, HEXOSIDES AND HEPTOSIDES (31)



X	Mannoside Series		Glucoside Series		Galactoside Series	
	α	β	α	β	α	β
-H	6.07	8.08	4.55	4.75	1.73	1.42
$-CH_3$	4.01	5.95				
$-CH_2OH$	1	1	1	1	1	1
$-CHOHCH_2OH$	0.55	0.51				

Decreased rate of glycoside hydrolysis due to electrophilic induction of the carboxyl group has been claimed on the basis of a twentyfold increase in hydrolysis rate when aldobiouronic acid (I) was reduced to 2-O-(4-O-methyl-α-D-glucopyranosyl)-D-xylitol (32). However, recent data on a comparison of methyl-α-D-glucoside and methyl-α-D-glucuronide indicate that the rate of glucoside hydrolysis is only twice that of the uronoside (33, 34).

Hamilton and Thompson (7) suggested that the absence of the isomeric aldotriouronic acid (III) was due to the stabilizing effect of the carboxyl being transmitted to glycosidic bonds further removed from the uronic acid moiety. Rånby and Marchessault (35) also suggested that the inductive effect of the 4-O-methylglucuronic acid would extend beyond the xylose-glucuronic acid linkage in 4-O-methylglucuronoxylan. Their hypothesis included a novel feature of an "activation" and "stabilization" of the glycosidic linkages in the xylan chain. Thus, B-C and B-E would be "stabilized" and A-B would be "activated" by the inductive effect of the uronic acid carboxyl. The result would be the formation of aldotriouronic acid EBC (II) (Fig. 7).

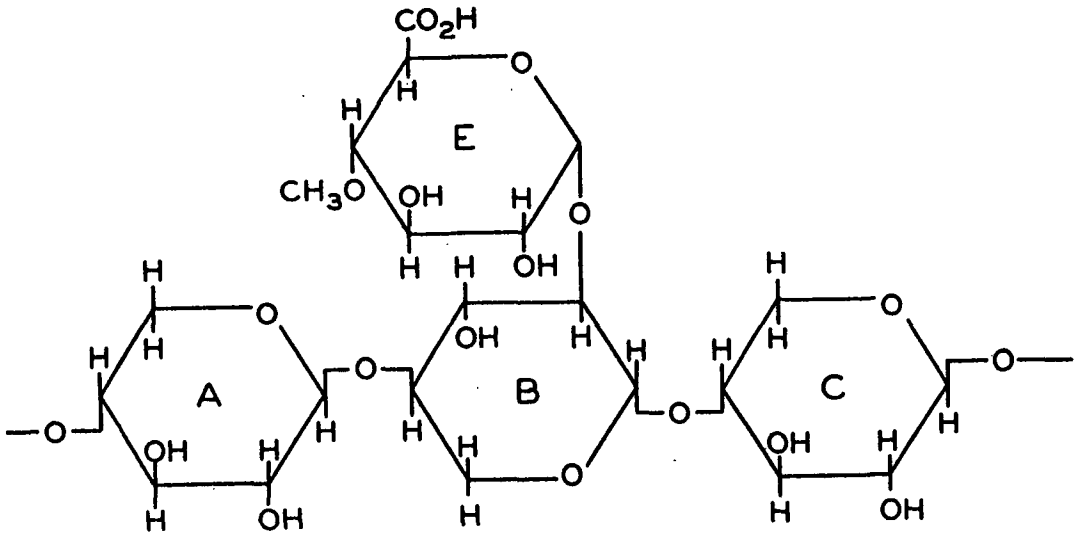


Figure 7. 4-O-Methyl Glucuronoxylan

PRESENTATION OF THE PROBLEM

The hypotheses of Hamilton and Thompson (7) and of Rånby and Marchessault (35) indicate that the inductive effect of the carboxyl in 4-O-methylglucuronoxylan would extend to the glycosidic linkages of the xylan chain. Reduction of the carboxyl, followed by graded acid hydrolysis of the reduced polymer was expected to provide a test of the above hypotheses. Two isomeric trisaccharides (IV, V) (Fig. 8) were expected after isolation of the hetero-trisaccharide portion.

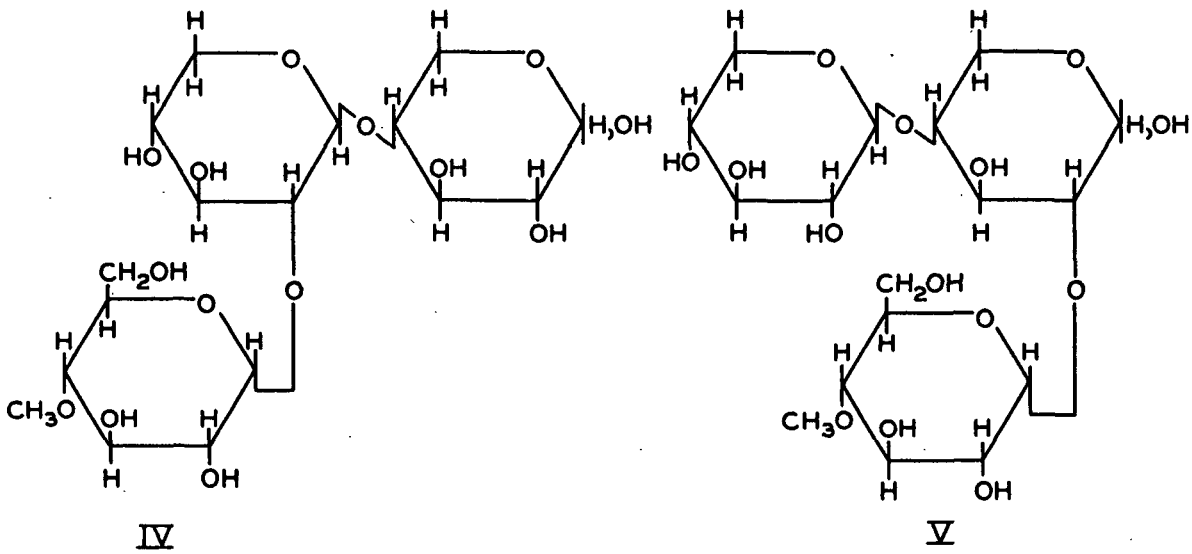


Figure 8. Neutral Hetero-Trisaccharides

A nearly equal mixture of the two isomers would be indicative of equal sensitivity of xylan glycosidic linkages and, hence, tend to confirm the above hypotheses. If isomer (IV) [having the same skeletal structure as ubiquitous aldotriouronic acid, (II)] predominates, this

would indicate that other factors beside carboxyl inductive effects are important in the hydrolysis of glycosidic linkages along the xylan chain.

Isolation of isomer (V) in sufficient quantity for characterization would be the first direct evidence for the currently accepted structure of 4-O-methylglucuronoxylan.

EXPERIMENTAL RESULTS

ISOLATION OF ELM 4-O-METHYLGLUCURONOXYLAN

American elmwood was chosen as a source of 4-O-methylglucuronoxylan because it has a low xylose-to-uronic acid ratio (7 compared with about 10-11 for most hardwoods) (17). Another advantage in using elm is that the xylan chain is not branched (cf. Table I). Disadvantages of elm are its low xylan content compared with that of most hardwoods (11% vs. 16-24%) and its slight content of unmethylated glucuronic acid residues (13). The 4-O-methylglucuronoxylan from elm has been characterized by Gillham and Timell (13).

Five American elm (Ulmus americana) trees, averaging 3-1/2 inches d.b.h., were selected from the Institute's ravine plantation in May, 1960. After the logs were peeled and chipped, a crude fractionation of sapwood from heartwood was made by water flotation. The sapwood portion was hand-picked or cut from the heartwood. Sapwood chips were reduced in size by passing them through the 12-inch Sprout-Waldron refiner with coarse plates. Reduction to sawdust was accomplished in the No. 1 Wiley mill. Every attempt was made to keep the sawdust from drying out, since even air-drying has been shown to reduce the amount of hemicellulose directly extractable by alkali (36).

The sawdust (54% ovendry) was pre-extracted twice with 70% ethanol (1000 ml./100 g. o.d. wood) for twelve hours at room temperature to remove extractives. After a thorough washing with deionized water, the sawdust was pre-extracted with one-tenth normal sodium hydroxide (1500

ml./100 g. o.d. sawdust) for four hours at room temperature to remove soluble lignin (37), pectic materials (38) and to saponify most of the acetyl. The sawdust was washed thoroughly with deionized water before extraction with strong alkali.

Potassium hydroxide was chosen over sodium hydroxide for the extraction because 4-O-methylglucuronoxylans have a high solubility in potassium hydroxide (39-41) and because potassium acetate is appreciably soluble in alcohol (42). A series of small-scale preliminary experiments were conducted to ascertain the proper concentration of potassium hydroxide for use in the large-scale isolation of 4-O-methylglucuronoxylan. The potassium hydroxide extractions were performed at room temperature for two hours with one liter of potassium hydroxide solution per 100 g. o.d. wood. The potassium hydroxide extracts were poured into two volumes of cold 95% ethanol containing sufficient acetic acid to bring the pH to about 6.0. The precipitated hemicellulose was washed and solvent exchanged with 80% ethanol, 95% ethanol, absolute ethanol, and petroleum ether (30-60°C.) before drying *in vacuo* over calcium chloride. The results obtained are recorded in Table V.

The 70% ethanol extract contained no detectable saccharide material. Hydrolysis of the polysaccharide precipitated by alcohol from the 0.1N sodium hydroxide extract gave galacturonic acid, galactose, xylose, and rhamnose, with traces of glucose, mannose, and arabinose by qualitative paper chromatography. All of the potassium hydroxide-extracted hemicellulose preparations after hydrolysis and qualitative paper chromatographic separation (in solvents A, B, and C, described fully in Appendix

I) contained xylose and uronic acids (aldobio and aldotrio) as the major components.

TABLE V

EFFECT OF POTASSIUM HYDROXIDE CONCENTRATION ON HEMICELLULOSE EXTRACTED FROM ELM

Sample no.	6	7	8
70% Ethanol extract, yield % on o.d. wood		1.5	
70% Ethanol extract, pH		8.1	
0.1N NaOH extract, yield % on o.d. wood (pptd. by ETOH)			0.2
0.1N NaOH extract, pH	11.9	11.8	11.8
Concentration of KOH solution, % by weight	5	10	24
Yield of hemicellulose, % on o.d. wood	7.4	13.0	13.8
Analysis of hemicellulose			
Moisture, %	8.45	7.94	7.56
Sulfated ash as K, %	4.17	6.95	7.77
CO_2[a], %	3.16	2.77	2.69

Calculation of Xylose/4-O-Methylglucuronic Acid Ratio

Basis : 100 g. crude hemicellulose

$\underline{a}$ = g. K as K in COOK	$\underline{a}$ =	x (39.1/44.0)
$\underline{b}$ = g. K as $KC_2H_3O_2$	$\underline{b}$ =	(% sulfated ash as K-$\underline{a}$)(98.14/39.1)
$\underline{c}$ = g. moisture		
$\underline{h}$ = g. pure hemicellulose	$\underline{h}$ =	100-$\underline{a}$-$\underline{b}$-$\underline{c}$
$\underline{x}$ = g. CO_2		

Sample no.	6	7	8
($\underline{a}$) = g. ash as K in COOK	2.81	2.46	2.39
($\underline{b}$) = g. ash as $KC_2H_3O_2$	3.41	11.27	13.50
($\underline{c}$) = g. moisture	8.45	7.94	7.56
($\underline{h}$) = g. pure hemicellulose	85.3	78.3	76.6
$\left(\frac{100x}{\underline{h}}\right)$ = % CO_2 on pure hemicellulose	3.70	3.54	3.51
xylose/4-O-methylglucuronic acid	~7 1/2	~8	~8
Yield pure hemicellulose, % on o.d. wood	6.32	10.1	10.5

[a]Uronic acid CO_2 by I.P.C. Method 25.

Trace amounts of galacturonic acid, galactose, glucose and mannose were also present in all samples. From the data in Table V, 10% potassium hydroxide was chosen for the large-scale extraction because of its good yield of 4-O-methylglucuronoxylan, low xylose/uronic acid ratio, and low acid requirement for neutralization.

Large-scale isolation of 4-O-methylglucuronoxylan was accomplished by use of the extractor shown in Fig. 9. About 1000 g. o.d. sawdust (at 53% o.d.) were charged to the extractor and treated in the same manner as in the small-scale extractions. The actual yield of crude hemicellulose was 98 grams or 9.8% on o.d. wood; however, since only part of the alkaline solution was expressed from the filter cake, the actual yield of hemicellulose would be about 13%. This compares favorably with the small-scale extractions. Analysis of the large batch of elm hemicellulose is given in Table VI.

Qualitative paper chromatographic examination of the large-scale hemicellulose hydrolyzate showed that the major components were xylose and aldobiouronic acid (I) with traces of galacturonic acid, galactose, glucose, and mannose. The data revealed that the large-scale batch of hemicellulose was similar in every respect to the small-scale batch.

Intrinsic viscosity of the 4-O-methylglucuronoxylan in molar cuene was $[\eta] = 0.978$. Using previous data (13) on elm 4-O-methylglucuronoxylan, the following calculation was made:

$$DP_n = K[\eta]$$

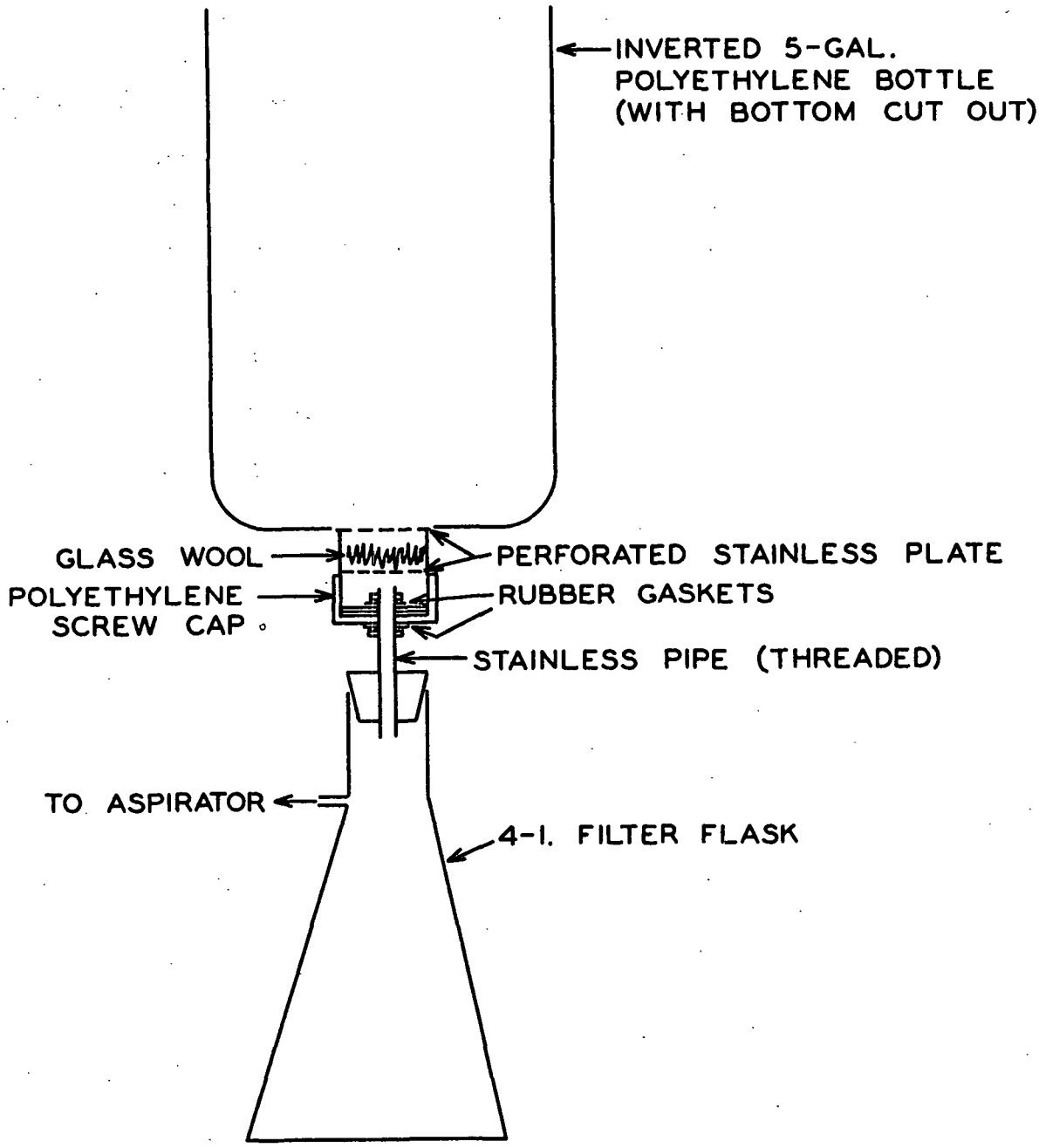


Figure 9. Apparatus for Large-Scale Isolation of Hemicellulose

where DP_n = 185 by osmotic pressure

$[\eta]$ = 1.113 in molar cuene

$$K = \frac{185}{1.113} = 166$$

Using K = 166, the 4-O-methylglucuronoxylan of this thesis would have a $D.P._n$ of 162 or slightly lower than the results ($D.P._n$ = 185) (18) for elm reported previously.

TABLE VI

ANALYSIS OF ELM HEMICELLULOSE

Moisture, %	9.22
Sulfated ash as K, %	2.85
CO_2, %	3.07

Calculated Results (as shown in Table V)

Basis: 100 g. crude hemicellulose

Ash as K in COOK	2.85 g.
Ash as K in $KC_2H_3O_2$	0.35 g.
Moisture	9.22 g.
Pure hemicellulose	87.72 g.
% CO_2 on pure hemicellulose	3.51
Xylose/4-O-methylglucuronic acid	~8

REDUCTION OF ELM 4-O-METHYLGLUCURONOXYLAN

Glaudemans and Timell (43) attempted to esterify and reduce (with sodium borohydride) the carboxyl groups in birch 4-O-methylglucuronoxylan. Their procedure was unsuccessful because of secondary methylation and poor reduction. Later Timell (23) checked for lactone formation by

treating birch O-acetyl-4-O-methylglucuronoxylan with aqueous sodium borohydride but found very little 4-O-methylglucose after hydrolysis of the "reduced" polymer.

Diborane, a Lewis acid, was shown to reduce rapidly carboxylic acids, aldehydes, and ketones but to reduce esters quite slowly (44-47). Smith and Stephan (48) applied the diborane reduction technique to simple acidic carbohydrate molecules and acylated acidic carbohydrate polymers. Their data (Table VII) showed that good reduction of carboxyl groups was accomplished.

TABLE VII

REDUCTION OF CARBOHYDRATE ACIDS BY DIBORANE (48)

Compound	Moles $NaBH_4$ per Mole COOH	Reduction or Yield of Reduced Compd., %
Methyl-α-D-galactopyranosiduronic acid	2.9	48
Methyl 2,3,4-tri-O-methyl-D-galacto-siduronic acid	2.9	56
Tetra-O-acetyl galactaric acid	5.8	75
Mesquitic acid acetate	2.9	--
Mesquitic acid acetate	14.5	90
Pectic acid acetate	2.9	nearly complete
Alginic acid propionate	14.5	82

Diborane was generated *in situ* by the addition of boron trifluoride etherate in bis(2-methoxyethyl) ether ("diglyme") to a solution of the compound and sodium borohydride in diglyme solution (45, 46, 48) where the following reaction takes place:

$$3NaBH_4 + 4BF_3{:}O(C_2H_5)_2 \rightarrow 2B_2H_6 + 3NaBF_4 + 4(C_2H_5)_2O$$

Since it was considered necessary to keep the compound to be reduced in solution [cf. (48)], the fully acetylated 4-O-methylglucuronoxylan was prepared according to the method of Carson and Maclay (49). Three 12.0-g. portions of elm hemicellulose were weighed into three 1000-ml. Erlenmeyer flasks. Formamide (200 ml.) was added to each flask, and the flasks were shaken until the hemicellulose was dispersed (2 hours). Pyridine (400 ml./flask) was added, followed by the addition of acetic anhydride (300 ml.) over a three-hour period with shaking. After 14 hours at room temperature, the reaction was terminated by pouring the contents of each flask into three liters of ice and water. Particle size of the gel was reduced by treatment in a Waring Blendor for one minute. After washing with 1-1/2 liters of cold 2% hydrochloric acid and cold water until neutral to litmus, the acetate was solvent exchanged through 95% ethanol, absolute ethanol, and petroleum ether (30-60°C.) before drying in vacuo over calcium chloride. Analysis of the acetylated hemicellulose is recorded in Table VIII.

TABLE VIII

ANALYSIS OF ACETYLATED ELM 4-O-METHYLGLUCURONOXYLAN

Sulfated ash	= 0.03%
Moisture	= 2.38%
Acetyl (50)	= 37.2%
Acetyl corrected for ash and moisture	= 38.1%
Acetyl corrected for ash, moisture, and free acid	= 37.2%
Theoretical acetyl content	= 38.3%
Yield of pure hemicellulose corrected for acetyl	= 90.3%

The reduction of the acetylated elm 4-O-methylglucuronoxylan was accomplished in the apparatus shown in Fig. 10. The dosage of sodium borohydride was 20 moles/carboxyl or 13.3 moles of diborane/carboxyl. This is a considerable excess of diborane over the theoretical requirement of one-half mole according to the following reaction (47):

$$RCO_2H + BH_3 \rightarrow [RCH_2OBO] + H_2 \xrightarrow{2H_2O} RCH_2OH + H_3BO_3$$

The volume of diglyme used in the reaction vessel was dependent on the solubility of sodium borohydride diglyme (51, 52). A list of reagents and their means of purification for diborane reduction are given in Appendix II. The system was purged with dry nitrogen before addition of reactants, and a flow of ten liters per hour was maintained during the reaction to sweep out excess diborane. The excess diborane was absorbed in acetone traps.

When the sodium borohydride (17.0 g.) was completely dissolved in diglyme (440 ml.), the acetylated 4-O-methylglucuronoxylan (45.5 g.) was added and stirred for three hours. The acetylated polymer did not dissolve in the diglyme-borohydride solution, but it did appear to be swollen. Boron trifluoride etherate (75.3 ml.) in 50 ml. diglyme was added dropwise over a four-hour period. Stirring was continued for an additional three hours and then the reaction mixture was allowed to stand overnight. After purging the system with nitrogen, the reaction mixture was poured into 200 ml. ice water. Large quantities of hydrogen were evolved and the following reactions were probably taking place:

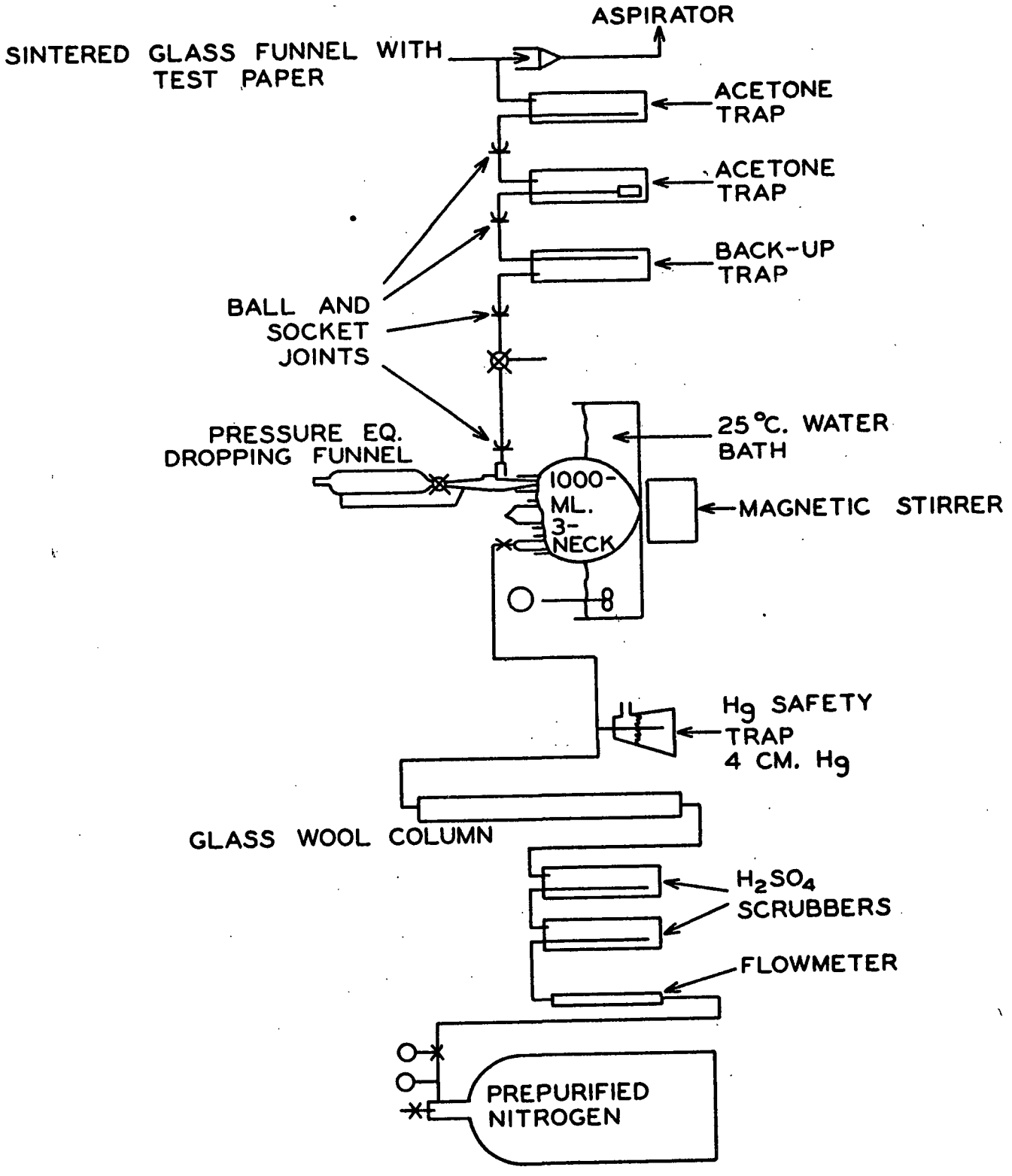


Figure 10. Diborane Reduction Apparatus

$$1/2\ B_2H_6 + 3H_2O \longrightarrow H_3BO_3 + 3H_2$$

$$BH_4^- + 2H_2O \longrightarrow BO_2^- + 4H_2$$

$$BF_3{:}O(C_2H_5)_2 + 3H_2O \longrightarrow H_3BO_3 + 3HF + (C_2H_5)_2O$$

The pH of the solution was 3.1, so 70 ml. of one-half normal sodium hydroxide was added to bring the pH to 7.0. The neutralized reaction mixture was poured into two volumes of 95% ethanol, and the reduced, partially acetylated 4-O-methylglucuronoxylan was removed by filtration. The precipitate was dissolved in 1000 ml. of 5% potassium hydroxide and heated for 45 minutes at 55°C. to deacetylate the polymer. After filtering through a fritted glass filter, the alkaline solution of the polymer was poured into two volumes of cold 95% ethanol containing sufficient acetic acid to bring the pH to 5.9. The deacetylated polymer gave a negative ferric hydroxamate test for esters (53). Analysis of the reduced deacetylated 4-O-methylglucuronoxylan follows: moisture, 2.39%; sulfated ash calculated as potassium acetate, 1.25%; carbon dioxide, 0.84%; and calculated pure 4-O-methylglucoxylan, 96.4%. Based on ash and moisture-free hemicellulose from the acetate, the yield of reduced hemicellulose was 90.1%. Over-all yield of 4-O-methylglucoxylan based on initial ash and moisture-free hemicellulose was 81.4%. Chromatographic examination (in solvents A and B, Appendix I) of the total hydrolysis of the 4-O-methylglucoxylan showed that xylose and 4-O-methylglucose were the predominant constituents but that a trace of galactose remained. The reduction efficiency was 75% of the carboxyl based on carbon dioxide evolution from the uronic acid test (I.P.C. Method 25). Since Yundt (54) found 0.50% carbon dioxide from xylose, a correction made for this non-uronic acid carbon dioxide would give about 88% reduction. The

reduction must have been nearly complete because no aldobiouronic acid was observed in the hydrolyzate.

The conditions used for total hydrolysis of the hemicellulose preparations (Appendix I, Procedure 1) were shown to produce very little 4-O-methylglucuronic acid. This is contrasted with the results of other investigators who used 72 or 77% sulfuric acid (13, 1) and found large quantities of 4-O-methylglucuronic acid in their hydrolyzates. One explanation may be found in the fact that very strong mineral acids readily hydrolyze uronosides (33).

The intrinsic viscosity of the reduced 4-O-methylglucuronoxylan was $[\eta] = 1.028$ or D.P. = (166) (1.028) = 171. This result indicates that the reduction procedure does not have any detrimental effect on the degree of polymerization of the hemicellulose. In fact, the slightly higher D.P. may be due to removal of lower molecular weight material in the many operations used during reduction.

GRADED ACID HYDROLYSIS OF REDUCED 4-O-METHYLGLUCURONOXYLAN

Since the activation energy for hydrolysis of the α-glycosidic bond is generally higher than that of the β-glycosidic bond (cf. Table III), lower hydrolysis temperatures should favor the preservation of the 4-O-methylglucose branches. Hydrolyses were run at 70 and 90°C. in sealed ampules containing 0.1 g. reduced hemicellulose and 2.00 ml. of normal sulfuric acid. After neutralization with barium carbonate or barium acetate, the supernatant liquors were chromatographed and evaluated quantitatively. The hydrolysis of 4-O-methylglucose branches was

determined by the method of McFarren, et al. (55) (Appendix I, Procedure 2), where the control sample was a total hydrolysis of the reduced 4-O-methylglucuronoxylan. Maximum yield of new trisaccharide was determined by measuring the relative optical density of the trisaccharide spot on a paper chromatogram according to the method of Jeffery, et al. (56) (Appendix I, Procedure 3a). The results obtained are listed in Table IX.

TABLE IX

GRADED ACID HYDROLYSIS OF REDUCED 4-O-METHYLGLUCURONOXYLAN

Yield of 4-O-methylglucose

Chromatogram No. 69

70°C.	4 hrs.	8 hrs.	12 hrs.	16 hrs.
% of Total	14	20	27	40

Chromatogram No. 87

90°C.	1/2 hr.	1 hr.	2 hrs.
% of Total	18	34	80

Trisaccharide Yield[a]

Chromatogram No. 67

70°C.	4 hrs.	8 hrs.	12 hrs.	16 hrs.
Relative density	0.56	1.00	0.87	0.92

Chromatogram No. 88

90°C.	1/2 hr.	1 hr.	2 hrs.
Relative density	0.84	0.84	0.37

[a] Estimated by spot density relative to 70°C. hydrolysis for eight hours.

The data of Table IX indicated that the maximum yield of trisaccharide would be obtained by hydrolyzing the 4-O-methylglucoxylan for eight hours at 70°C. in 1.00N sulfuric acid.

Chromatographic examination of the eight-hour hydrolyzate (in Solvent B) showed that a new homologous carbohydrate series was present (see Fig. 13, and Table XIII). Isolation of the new di-, tri-, and tetrasaccharide materials by paper chromatography gave substances which, in each case, were found to contain xylose and 4-O-methylglucose after complete hydrolysis.

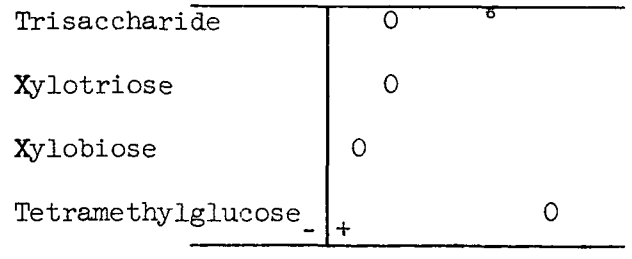
The reduced elm 4-O-methylglucuronoxylan (20.5 g.) was placed in a 500-ml. Erlenmeyer flask to which was added 420 ml. of 1.00N sulfuric acid. The stoppered flask was kept in a 70.0°C. bath for eight hours. After cooling and centrifuging, the supernatant hydrolyzate was neutralized with barium hydroxide solution to pH 5.5. After removal of barium sulfate by filtration, the hydrolyzate was sorbed on a carbon-celite column treated with stearic acid (57) (this column was 2 by 36 inches packed with 1550 cc. of absorbent containing about 400 g. charcoal and 400 g. celite). The column was washed with two liters of distilled water prior to starting the gradient elution. Gradient elution was performed by the technique of Alm, et al. (58). The pressure on the system was held constant at 5 p.s.i. and the flow rate allowed to vary from 2-1/2 - 1 ml./min. as the gradient changed. Fraction cutter time intervals were varied so as to give 15-20 ml. fractions. Every fifth fraction was monitored by paper chromatography in Solvent B. The desired trisaccharides (IV + V) were found in fractions corresponding to a gradient of 5-13% ethanol. These fractions also contained a large quantity of xylotriose, so that a subsequent separation by paper chromatography was necessary. The fractions containing the desired trisaccharides were combined and concentrated in a cyclone evaporator under reduced pressure.

The sirup was streaked on four 24-inch wide sheets of Whatman 3MM paper and developed 84 hours in Solvent B. Guide strips were cut and the desired trisaccharide was eluted, cleared through asbestos, and concentrated. A chromatographically pure sample was obtained by this procedure.

The chromatographically pure trisaccharide was subjected to paper electrophoresis in 0.1M borate (pH 9.2) at 700 volts for 4-1/2 hours. After spraying with p-anisidine (see Appendix I), two distinct spots were observed. The major component (about 95% by visual inspection) had the greater mobility.

TABLE X

PAPER ELECTROPHORESIS OF OLIGOSACCHARIDES



	Movement from Starting Line, cm.	M_G [a]
Major trisaccharide component	2.3	0.136
Minor trisaccharide component	6.0	0.061
Xylotriose	1.9	0.144
Xylobiose	0.8	0.166
Tetramethylglucose	9.0	0.00

[a] M_G = Movement of sugars relative to true movement of glucose calculated from $\frac{\text{glucose movement}}{\text{tetramethylglucose movement}} = \frac{11.2 \text{ cm. } (-)}{2.5 \text{ cm. } (+)}$

The most important structural feature governing electrophoretic movement for the xylose oligosaccharide series will be the point of attachment of the remainder of the molecule to the reducing moiety (59). Thus, neutral trisaccharide (IV) will be expected to have a mobility similar to xylotriose, while the isomer (V) will have very little electrophoretic movement because the hydroxyl on C_2 of the reducing moiety is not available for complexing.

The above electrophoretic results indicate that the structure of the major trisaccharide is similar to that of the ubiquitous aldotriouronic acid. Therefore, it was desirable to attempt to prepare the new trisaccharide (IV) from aldotriouronic acid (II).

PREPARATION OF A NEW NEUTRAL TRISACCHARIDE FROM ALDOTRIOURONIC ACID

Preparation of this new neutral trisaccharide was based on the hypothesis that the acidic nature of diborane would not cleave an acetyl group blocking the reducing end of the completely acetylated aldotriouronic acid, but that it would rapidly reduce the exposed carboxyl group.

Aldotriouronic acid* (II) (0.51 g.) from aspen was refluxed with acetic anhydride (30 ml.) and anhydrous sodium acetate (0.35 g.). The reaction mixture was poured into ice water and an oily mass was obtained. Chloroform extraction, followed by removal of chloroform by concentration

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* Courtesy of Mr. E. E. Dickey.

in vacuo, gave 0.85 g. of acetate. After dissolving the acetate in purified tetrahydrofuran (cf. Appendix II), the solution was placed in a gas washing bottle supplied with a sintered-glass gas disperser. This bottle was fitted with ball and socket ground-glass joints and was inserted into the apparatus shown in Fig. 10 in the train after the reaction flask.

Diborane was generated in the reaction flask by addition of sodium borohydride in diglyme to a diglyme solution of boron trifluoride etherate (45-47). Chemicals were charged to the generator to give 16 moles of diborane/carboxyl. Diborane was swept out of the generator into the tetrahydrofuran by a stream of dry nitrogen. Diborane was generated over a 45-minute period, and the total reaction time was 3-3/4 hours. The reaction was terminated by addition of methanol, and the diborane was decomposed slowly overnight. The solvents were removed by concentration in vacuo at 35°C. Three successive 30-ml. portions of methanol were added and evaporated to remove methylborate (yield product 0.66 g.). Deacetylation with barium methylate (60) was followed by neutralization with sulfuric acid and removal of barium sulfate. Concentration in vacuo gave a sirup (0.51 g.) which gave a negative ferric hydroxamate test for esters (53).

Resolution of the crude sirup on Whatman 3MM paper in Solvent B gave 0.30 g. sirup of a new neutral trisaccharide which was assumed to be O-α-4-O-methyl-D-glucopyranosyl-(1⟶2)-O-β-D-xylopyranosyl-(1⟶4)-D-xylopyranose based on the previously determined structure of the ubiquitous aldotriouronic acid (7-9). The chromatogram indicated that

unreduced acid, xylobiose, xylose, and 4-O-methylglucose were also present in the reaction product. Since these sugars were not present as their alditols it was assumed that the hydrolysis took place after reduction, possibly during evaporation (or during acetylation).

The new neutral trisaccharide was shown to be chromatographically pure (on paper in Solvent B) and electrophoretically pure (on paper in 0.1M sodium borate).

After many attempts to effect crystallization, the sirup was taken up in hot 85% methanol, chilled, and a small quantity of dark material which precipitated was removed by filtration. When the mother liquor was chilled and concentrated *in vacuo* at 35°C., a large quantity of white crystals formed on the sides of the flask. The crystals appeared to be very hygroscopic since they coalesced in about 15 minutes after the vacuum was released and the small quantity of mother liquor wetted them. Further attempts at crystallization failed.

A portion of the trisaccharide sirup (20 mg.) was converted into a crystalline phenylosazone. After recrystallization from 60% ethanol, the decomposition point was 240-241°C.

COMPARISON OF TRISACCHARIDE FROM GRADED ACID HYDROLYSIS OF REDUCED 4-O-METHYLGLUCURONOXYLAN WITH NEW NEUTRAL TRISACCHARIDE PREPARED FROM ALDOTRIOURONIC ACID

A side-by-side comparison of the hetero-trisaccharide portion ("unknown") from the graded acid hydrolysis of reduced 4-O-methylglucuronoxylan with the new neutral trisaccharide ("known") from aldotriouronic

acid revealed that they gave identical spots after paper chromatography in Solvent B. Paper electrophoresis in 0.1M sodium borate gave the results in Table XI.

TABLE XI

PAPER ELECTROPHORESIS OF NEUTRAL TRISACCHARIDES

Xylotriose		0	
Trisaccharide from graded hydrolysis		0	
Trisaccharide from aldotriouronic acid		0	
Tetramethylglucose[a]	+	-	0

	Movement from Starting Line, cm.	M_G
Xylotriose	3.5	0.145
Trisaccharides from graded hydrolysis		
Major component	3.3	0.147
Minor component	8.2	0.058
Trisaccharide from reduction of aldotriouronic acid	3.5	0.145
Tetramethylglucose	12.0	0.000

[a] Courtesy of Dr. N. S. Thompson.

These results indicate that the major trisaccharide component from the graded acid hydrolysis of reduced 4-O-methylglucuronoxylan might be identical with the "known" neutral trisaccharide prepared from aldotriouronic acid.

The optical rotations of the "known" and "unknown" trisaccharides were measured after first drying the sirups to a froth *in vacuo* over calcium chloride.

known trisaccharide $[\alpha]_D^{25} = +45.7$ ($c = 6.7$, water)

unknown trisaccharide $[\alpha]_D^{25} = +34.0$ ($c = 7.2$, water)

The difference in rotation may be due to the mixture of trisaccharides in the unknown plus the uncertainty of concentration especially in the case of the unknown.

Positive proof of identity was made by a comparison of the crystalline osazones. The small quantity of isomeric trisaccharide in the graded hydrolysis sample ("unknown") should not seriously interfere in the osazone preparation since it should not form an osazone. Phenylosazones were prepared as follows:

	Trisaccharide from Aldotriouronic Acid "Known"	Trisaccharide from Graded Acid Hydrolysis "Unknown"
Trisaccharide, g.	0.160	0.223
$NaC_2H_3O_2 \cdot 3H_2O$, g.	0.450	0.735
Phenylhydrazine HCl, g.	0.300	0.490
Water, ml.	3.5	5.0
Heat for 30 minutes in boiling water bath		
Precipitate formation, min.	12	14
Yield of osazone, g.	0.0567	0.0518
Recrystallize from hot 60% ethanol		
Yield of recrystallized osazone, g.	0.394	0.0473

The low yields of osazone were due in part to large mechanical losses. In addition, the initial sugar concentration was estimated by drying the sirups to a froth in vacuo and, hence, the actual concentration was probably somewhat less than the figures given.

The osazones were dried in an Abderhalden drier in vacuo with phosphorous pentoxide over boiling acetone for 1-1/2 hours prior to microanalysis.*

TABLE XII

ANALYSIS OF TRISACCHARIDE PHENYLOSAZONES

		Found	
Analysis	Calculated	"Known" Osazone	"Unknown" Osazone
C_{29}	54.71	54.9	53.9
H_{40}	6.33	6.2	6.2
O_{12}	30.16		
N_4	8.80	8.9	8.8
OCH_3	4.88	4.0	3.9

Chemical analysis established the two phenylosazones as identical with each other and in agreement with the calculated values for the new trisaccharide phenylosazone. Melting points of the two osazones were identical at 240-241°C. (m.p. tubes inserted at 210°C. and heated at a rate of 8°C./min.). For all practical purposes, the infrared absorption spectra were identical. X-ray diffraction patterns, Fig. 11, reveal that the crystalline osazones are identical.

* Microanalyses by Huffman Microanalytical Laboratories, Wheatridge, Colorado.

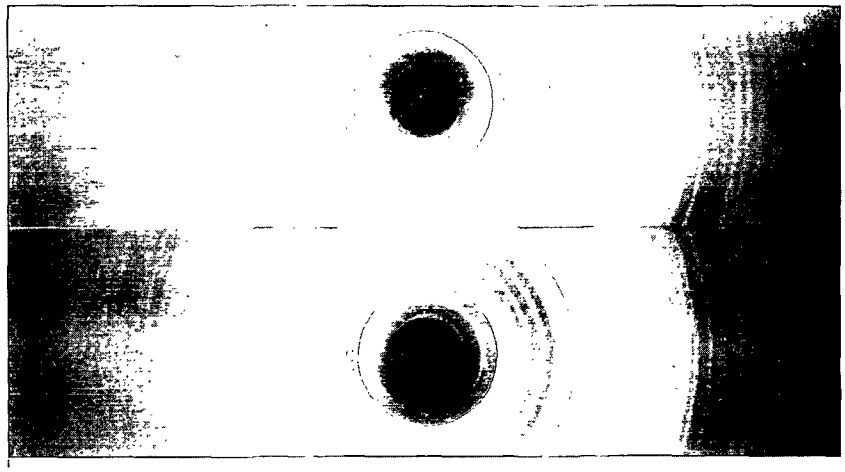

Figure 11. X-ray Diffraction Patterns of Phenylosazones

Evidence for a trisaccharide is obtained from the nitrogen and methoxyl analysis of the osazones. Further evidence was obtained by an analysis of the xylose/4-O-methylglucose ratio after hydrolysis of the trisaccharides. Methyl-4-O-methyl-α-D-glucopyranoside*, xylose and the "known" and "unknown" trisaccharides were hydrolyzed with one-half normal hydrochloric acid, neutralized with silver carbonate, and spotted for quantitative chromatography according to Appendix I, Procedure 2.

For "known" trisaccharide: xylose/4-O-methylglucose = 1.45

For "unknown" trisaccharide: xylose/4-O-methylglucose = 1.67

When the trisaccharide hydrolyzates were chromatographed in Solvent D, no xylitol was found in either the "known" or "unknown" hydrolyzate.

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* Courtesy of Dr. F. Smith, University of Minnesota.

This finding indicated that the xylose/4-O-methylglucose ratios (and rotations of trisaccharides) are not influenced by xylitol. It also indicated that the reducing group was completely protected during reduction.

COMPARISON OF THE GRADED ACID HYDROLYSIS OF REDUCED 4-O-METHYLGLUCURONOXYLAN WITH UNREDUCED 4-O-METHYLGLUCURONOXYLAN

Approximately 0.100-g. samples of reduced and unreduced 4-O-methylglucuronoxylan were placed in small ampules along with 2.00 ml. of normal sulfuric acid. After sealing, the ampules were heated for eight hours at 70.0°C. Following neutralization with barium acetate, the samples were spotted quantitatively and chromatographed in Solvent A for the unreduced sample and in Solvent B for the reduced sample. Known quantities of aldotriouronic acid (for unreduced sample) and new trisaccharide prepared from aldotriouronic acid (for reduced sample) were also spotted and served as controls. Quantitative chromatographic Procedure 3b (Appendix I) was used to estimate the amounts of aldotriouronic acid and neutral trisaccharides present. The yield of aldotriouronic acid was found to be approximately 7%, while the yield of new neutral trisaccharide was approximately 4%. The low yield of neutral trisaccharide was due primarily to the acid lability of the 4-O-methylglucose branches. No 4-O-methylglucuronic acid was detected in the hydrolyzate from the unreduced sample.

A comparison of the chromatograms of the acidic and neutral homologous series is seen in Fig. 12. There is a marked similarity between

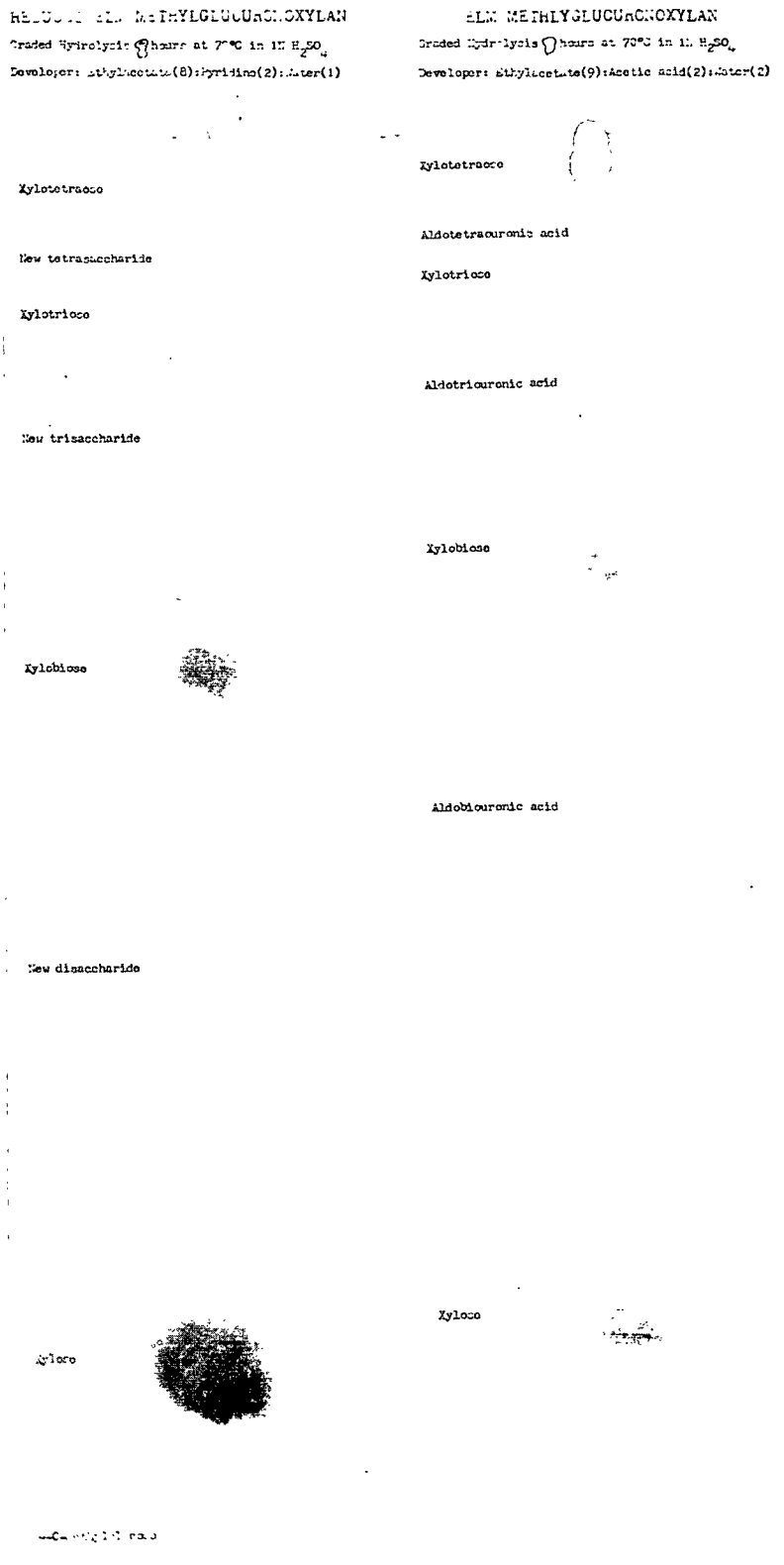

Figure 12. Chromatograms of Hydrolyzates, Eight Hours at 70°C. in 1.00N Sulfuric Acid

the oligosaccharides obtained from the graded acid hydrolysis of the reduced and unreduced polymers. Movement of the sugar components relative to xylose is given in Table XIII.

TABLE XIII

R_x VALUES OF COMPONENTS FROM GRADED ACID HYDROLYSIS OF REDUCED AND UNREDUCED 4-O-METHYLGLUCURONOXYLAN

Reduced Polymer Solvent B	R_x	Unreduced Polymer Solvent A	R_x
New tetrasaccharide	0.04	Aldotetraouronic acid	0.06
Xylotriose	0.08	Xylotriose	0.09
New trisaccharide	0.14	Aldotriouronic acid	0.17
Xylobiose	0.30	Xylobiose	0.32
New disaccharide	0.54	Aldobiouronic acid	0.49
Xylose	1.00	Xylose	1.00
4-O-Methylglucose	1.23	4-O-Methylglucuronic acid	no spot

The above results when plotted as log R_x vs. D.P. of saccharide indicate by their linear relationships that two homologous series of saccharides are present in each of the hydrolyzates. These results are shown in Fig. 13.

The reduced 4-O-methylglucuronoxylan hydrolyzate contains both the xylodextrin series and a heterodextrin series composed of xylose and 4-O-methylglucose. The unreduced 4-O-methylglucuronoxylan hydrolyzate contains the xylodextrin and oligouronide series as previously reported (7).

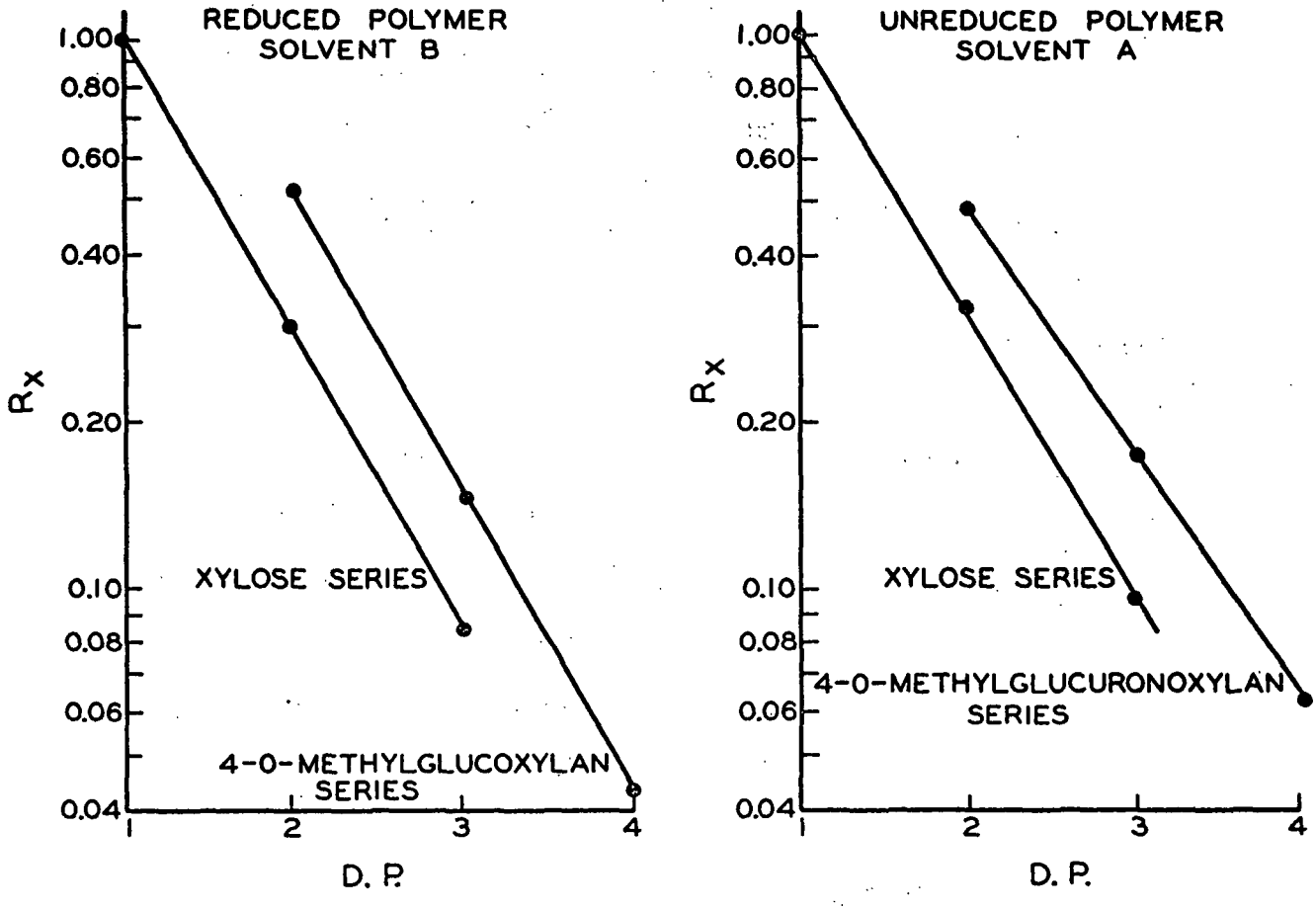

Figure 13. Log R_x vs. D.P. for Saccharides from Graded Acid Hydrolysis of Reduced and Unreduced 4-O-Methylglucuronoxylan

DISCUSSION OF RESULTS AND CONCLUSIONS

A relatively pure 4-O-methylglucuronoxylan was isolated from American elm by direct extraction of the wood with 10% potassium hydroxide. Reduction of the uronic acid carboxyl group by diborane was accomplished without any apparent decrease in the degree of polymerization of the sample. This is the first successful reduction of carboxyl group to be reported for any wood hemicellulose.

Graded acid hydrolysis of the reduced and unreduced 4-O-methylglucuronoxylan showed that the yield of heterotrisaccharide from the reduced polymer amounted to 55% of the aldotriouronic acid produced from the acidic polymer. This difference is probably due to the stability of the α-1, 2 glycosidic linkage in the unreduced polymer. No hydrolysis of this bond was detected in the unreduced polymer, while about 20% of the total α-1, 2 branch linkages were hydrolyzed at maximum trisaccharide yield for the reduced polymer.

Data presented previously in the experimental section have shown that the major heterotrisaccharide component from the graded acid hydrolysis of reduced 4-O-methylglucuronoxylan has a skeletal structure identical with that of the ubiquitous aldotriouronic acid. The fact that the new neutral trisaccharide predominates about 20:1 over its isomer indicates that the hypotheses of Hamilton and Thompson (7) and Marchessault and Rånby (35) require modification. The high yield of new neutral trisaccharide and its similarity in structure to aldotriouronic acid would suggest that the inductive effect of the carboxyl probably does not extend to the glycosidic linkages of the xylan chain.

An alternate hypothesis based on conformational resistance will be presented (cf. Appendix III). Assuming that glycosidic hydrolysis proceeds via the cyclic mechanism* and that formation of the carbonium ion necessitates a transformation from the puckered chair form to a planar half-chair form as suggested by Edward (28) (cf. Introduction), the large bulky substituent (4-O-methylglucose) on C_2 of the xylose moiety will provide resistance to conform to the half-chair form. This resistance would be sufficient to cause a lower rate of hydrolysis for glycosidic bond B-C relative to A-B as shown in Fig. 14 and by analogy to the data presented in Table V. The Hirschfelder model also indicates the possibility of some steric effect due to the proximity of the 4-O-methylglucose branch to the B-C glycosidic bond. This effect would exert itself primarily by hindering the approach of a proton to the glycosidic oxygen.

A new method of preparing neutral aldoses from their uronic acid analogues was developed. The method was based on the specificity of diborane for reduction of carboxyl groups. In this procedure, the reducing group was blocked by acetylation. The method was used to prepare a new compound, O-α-4-O-methyl-D-glucopyranosyl-(1→2)-O-β-D-xylopyranosyl-(1→4)-D-xylopyranose from the "ubiquitous aldotrio-uronic acid." The new trisaccharide could not be crystallized in a stable form, so its crystalline phenylosazone was prepared. The phenylosazone was characterized fully by analysis, melting point, x-ray diffraction pattern, and infrared spectrum.

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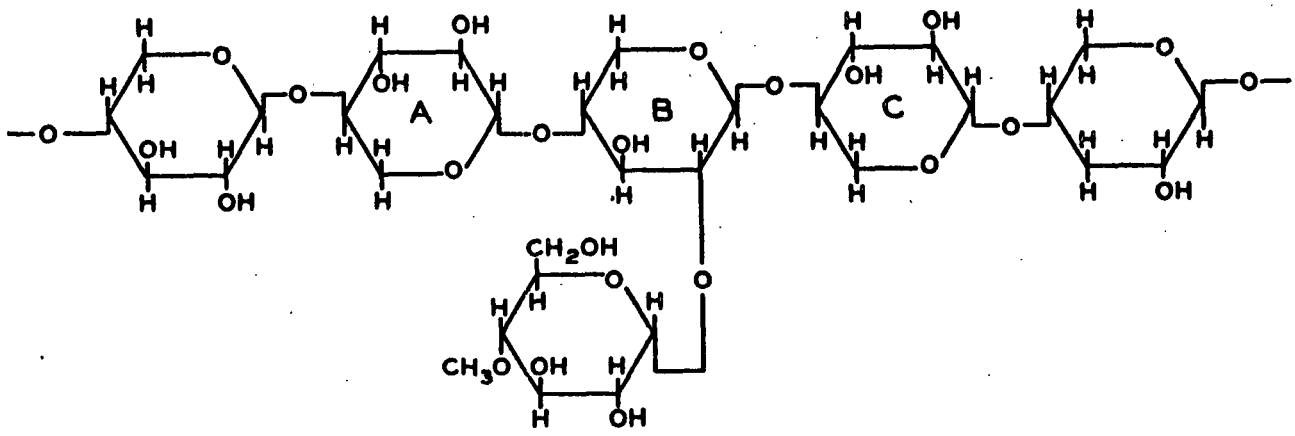
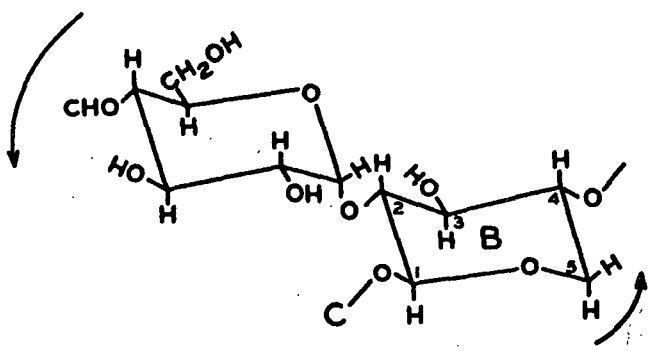
*Bunton and co-workers (61) mention unpublished results which support the cyclic pathway of glycoside hydrolysis.





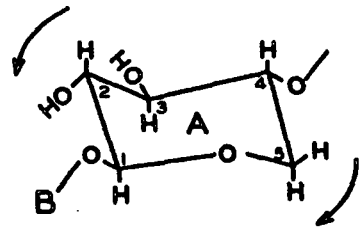


Figure 14. Reduced 4-O-Methylglucuronoxylan

ACKNOWLEDGMENT

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APPENDIX I

CHROMATOGRAPHIC PROCEDURES

DEVELOPERS

Solvent A for resolution of acidic substances--ethyl acetate (9):acetic acid (2):water (2).

Solvent B for resolution of neutral substances and holding acidic materials near the starting line--ethyl acetate (8): pyridine (2):water (1).

Solvent C for resolution of galacturonic and glucuronic acid--ethyl acetate (5):pyridine (5):water (3):acetic acid (1).

Solvent D for resolution of xylose from xylitol--butanol (10): pyridine (3):water (3).

Solvent E for quantitative determination of monosaccharides--ethyl acetate (8):pyridine (2):water (1):silver nitrate 0.15N.

SPOT DETECTION REAGENTS FOR SPRAYING OR DIPPING

p-Anisidine: dissolve 1.0 g. p-anisidine hydrochloride in 10 ml. water, add 20 ml. 95% ethanol, and finally add 170 ml. n-butanol.

p-Anisidine for paper electropherograms: make up solution the same as above but add 10.0 g. monochloroacetic acid.

Silver nitrate: dissolve 5.10 g. silver nitrate in 10 ml. water and add 190 ml. acetone. Add water dropwise if necessary to dissolve any precipitate.

Aniline: dissolve 2.0 g. monochloroacetic acid in 200 ml. anhydrous ether, then add 1.0 ml. freshly distilled aniline.

PROCEDURE 1. MICROHYDROLYSIS TECHNIQUE FOR MONOSACCHARIDES

The procedure is essentially that of Peat, et al. (62). Place 8 mg. hemicellulose in a 3-ml. test tube and add 0.15 ml. of 88% formic acid. Heat the mixture on a steam bath until the hemicellulose dissolves

and then add 0.30 ml. normal sulfuric acid and heat for three hours on a steam bath. Neutralize the sulfuric acid by adding 30 mg. barium carbonate, then centrifuge, and chromatograph the supernatant hydrolyzate.

PROCEDURE 2. QUANTITATIVE PAPER CHROMATOGRAPHY OF MONOSACCHARIDES

The procedure is essentially that of McFarren, et al. (55) with some additional refinements. To obviate nonuniform development due to flooding at torn edges, precut paper (Whatman No. 1 9-1/2 by 22 inches) was used. Sugar solutions were spotted so that the spot areas were uniform. Solvent E was used for resolution of the neutral monosaccharides. After development, the chromatogram was dried in the dark and then placed in a light-tight tank containing ammonia vapor. After one hour in the ammonia tank, the sheet was heated for five minutes at 105°C.

Transmission measurements with a 3-mm. diameter search unit or reflectance (red or yellow filter) measurements with a 0.25-inch diameter boot were made on the spots with a Welch Densichron. The background reading between the spots was averaged and set at 100% transmittance or reflectance so that the net reflectance or transmittance was measured. The minimum reflectance or transmittance of a given spot was obtained. The results were generally plotted as log concentration vs. net optical density, for which a linear relationship was usually found over a limited concentration range. The use of Kubelka Munk K/S vs. concentration (56) can also be used. Either method requires that the unknown sample be bracketed by spots of known concentration on the same sheet of paper. Normal precision is $\pm$ 5% for glucose and xylose.

PROCEDURE 3. QUANTITATIVE PAPER CHROMATOGRAPHY OF OLIGOSACCHARIDES OR ACIDIC MONOSACCHARIDES

The procedure is similar to that of Jeffery, et al. (56). The same precautions as to paper and to spot size given above were used in this procedure. Solvents A or B were used depending on the desired information. After drying, the chromatograms were dipped carefully in the aniline reagent. Spot development was obtained by heating the chromatogram at 105°C. The spot density was evaluated by two methods.

a. Reflectance or transmittance measurements with Densichron as described in Procedure 2.

b. Visual comparison with standards using transmitted light.

APPENDIX II

REAGENTS USED IN DIBORANE REDUCTION

SODIUM BOROHYDRIDE

Sodium borohydride with minimum purity of 98% was kindly supplied by Callery Chemical Company.

DIGLYME OR BIS(2-METHOXYETHYL) ETHER

Practical-grade diglyme from Eastman Organic Chemicals was purified by fractional distillation over calcium hydride at 159-160°C. Pure solvent was obtained by fractional distillation over lithium aluminum hydride at reduced pressure (51).

BORON TRIFLUORIDE ETHERATE

Purified-grade boron trifluoride etherate from Eastman Organic Chemicals was not further purified but it was noted that one of the two bottles obtained had darkened and would require distillation before use.

TETRAHYDROFURAN

Reagent-grade tetrahydrofuran was treated with potassium hydroxide pellets and allowed to stand one week. The tetrahydrofuran was decanted and then distilled. The distillate was treated with lithium aluminum hydride and the pure solvent was obtained after fractional distillation over lithium aluminum hydride at 65-66°C.

TEST PAPERS FOR DIBORANE

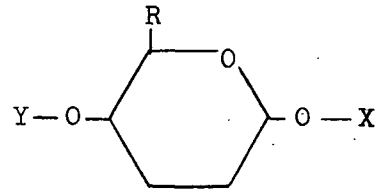
Since diborane is very toxic, with a threshhold limit of 0.1 p.p.m. for eight hours daily exposure (63), special test papers were used to detect its active hydrogen. Filter paper impregnated with triphenyltetrazolium chloride (0.5 g.) in pyridine (2.5 ml.)--water (2.5 ml.)--quinoline (19 ml.) (64) was placed on a sintered glass funnel. When gas was pulled through the test paper by an aspirator, a rapid formation of red diformazan dye indicated high concentrations of diborane.

APPENDIX III

NOTE ON THE INTERPRETATION OF THE DATA OF MARCHESSAULT AND RÅNBY

The experimental data of Marchessault and Rånby (35) can also be explained on the basis of strain and conformational influences.

In discussing the homogeneous hydrolysis (in 81.25% phosphoric acid) of unreduced and sodium borohydride-reduced bleached wood pulp, Marchessault and Rånby hypothesize an activating and a stabilizing inductive effect. The electrophilic group, R, activates linkage -O-Y and stabilizes linkage -O-X.



It was stated that R (i.e., carboxyl or carbonyl) would be equally effective at position 2 or 3. The data that they present to support their hypothesis indicated that the initial rate of hydrolysis for the unreduced pulp sample is 60% greater than for the reduced pulp sample. Furthermore, the reduced pulp sample had the same rate of hydrolysis as a sample of cotton linters. Their explanation for the rapid initial hydrolysis rate of the unreduced sample was activation of the -O-Y linkage due to the inductive effect of an electrophilic group (presumably a carbonyl, since carboxyl groups are not reduced by aqueous sodium borohydride).

Theander (65) found that methyl-β-D-glucopyranoside when oxidized with chlorine and/or hypochlorite produced methyl-β-D-2-oxo-glucopyranoside, methyl-β-D-3-oxo-glucopyranoside and methyl-β-D-6-oxo-glucopyranoside as neutral oxidation products. The order of hydrolysis in 0.5N sulfuric acid at 100°C. was methyl-3-oxo-glucoside > methyl-2-oxo-glucoside > methylglucoside (66).

In spite of the alkali sensitivity of 2- and 3-oxo-glucosides (66) and the fact that the wood pulp was preswollen in cuene for 30 minutes at 0°C., some 2- and 3-oxo-glucosides could exist in the unreduced preswollen wood pulp used by Marchessault and Rånby. If 2- and 3-oxo-glucosides constitute a large portion of the groups reduced by sodium borohydride in the pulp of Marchessault and Rånby, then their data can be explained by the greater acid lability of linkage -O-X in the unreduced pulp and its stabilization after reduction of oxo groups. The rapid hydrolysis of 2- and 3-oxo-glucosides can be explained by the strain introduced by the exo-double bond on a six-numbered ring (67).

The inductive effect of the carboxyl group must be altered by the use of strong phosphoric acid since it was shown that methyl-α-D-glucoside and methyl-α-D-glucuronoside hydrolyzed at about the same rate in 81.25% phosphoric acid (33).