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EXTRACTION AND STRUCTURAL STUDY

OF HEMICELLULOSE B FROM

TALL FESCUE (*FESTUCA ARUNDINACEA*)

A thesis presented in partial fulfilment of
the requirements for the degree of Master
of Science in Chemistry at
Massey University

Patana Hensanghongs

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SUMMARY

(1) Hemicellulose B has been extracted from Tall Fescue (*Festuca arundinacea*).

(2) The homogeneity of Branched Hemicellulose B was determined By Fractional Precipitation and electrophoresis.

(3) Gas-liquid chromatographic study showed that the polysaccharide contains xylose, arabinose and hexose.

(4) The uronic acid content was quantitatively determined by specific colour reactions.

(5) Quantitative analysis of the monosaccharides from the acid hydrolysis product by partition chromatography on cellulose column.

(6) Methylation by methods of Haworth, Kuhn and Purdie followed by hydrolysis yielded the following methyl ethers:
2,3-di-O-methyl-D-xylose,
2-O-methyl-D-xylose,
2,3,4-tri-O-methyl-D-xylose,
2,3,5-tri-O-methyl-L-arabinose.

(7) The methyl ether monomers were quantitatively determined by gas-liquid chromatography.

(8) From the results a simplified structure can be suggested for the branched hemicellulose B, consisting of 16β -D-xylo-pyranose residues linked together by 1-4 glycosidic bonds, and with a terminal side chain D-glucuronic acid residue linked through C-2 to the xylose unit. L-arabinose unit probably occurs as a side chain and is linked through position 3 to every fourth D-xylose unit.

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CHAPTER I

INTRODUCTION

1.1 Hemicellulose

The structural carbohydrates are mixtures of polysaccharides which together with lignin, constitute the cell wall. Usually they are divided into three fractions: pectic substances, hemicellulose and cellulose.[1]

Pectins, widely distributed in land plants are characterised by a main chain of 1 - 4 linked galacturonic acid units. They may also contain rhamnose, galactose, xylose, arabinose and fucose in varying amounts. These sugars form part of the main chain in some pectins and branch chains in others.[2] The pectic triad consists of galacturonan, araban and galactan. Pectic substances occur without exception in all higher plants. It is possible that pectic substances may serve as protective agents for natural rubber particles and may be important food reserve for the plant.[3] They are found most abundantly in the primary cell walls and in the intercellular layer.[4]

Cellulose is generally present in the plant cell as aggregates of fibrils or partly crystalline bundles. Chemical proofs indicate that cellulose is a linear chain of D-glucopyranose units connected uniformly by β-1,4 links.[5] Cellulose is the main constituent of the cell walls of land plants and serves as the

primary structural element.[4] The cellulose fibrils are usually embedded in other polysaccharide material e.g. the hemicellulose in the higher plants.[4] Cellulose may be obtained from bast fibers such as flax (80 - 90% cellulose), hemp (65 - 75% cellulose, jute (60 - 70% cellulose) and ramie (85% cellulose). Leaf fibers such as Manila Hemp, banana and sisal contain 50 - 70% cellulose. Whole leaves have a low cellulose content (most young leaves contain about 10% cellulose while older leaves contain 20% cellulose or more). Wood contains 40 - 50% cellulose. Agricultural residues such as corn stalks, corn cobs and wheat straw contain about 30% cellulose. Cellulose is also present to the extent of 1 - 20% in most sea weeds e.g. the lichenan of Iceland moss is also considered as cellulose.

The name hemicellulose was first proposed in 1891 by Schulze[6], who was examining the products extracted from leguminous seeds, brans and green tissues. He isolated a group of carbohydrates similar to cellulose which were characterised by their behaviour with dilute mineral acid, being more easily hydrolysed than cellulose. This group of polysaccharides are chemically and structurally related to cellulose, for which reason Schulze designated the group hemicellulose. The relationship between hemicellulose and cellulose is demonstrated in Fig. 1 which owes its origin to Norman[7] and Wise[8]. It is now known that the hemicelluloses are not precursors of cellulose and have no part in cellulose biosynthesis but rather represent a distinct

	Holocellulose	
	Hemicellulose	Cellulose
Ligin containing the non-carbohydrate material of the cell wall	Shorter chains containing: Xylose Units Mannose Units Uronic acid units Methoxyl groups Acetyl groups and (possibly galactose and arabinose units)	Longer chain length including: α - cellulose "True" cellulose β -d-glucopyranose units

Figure 1. Relation of Hemicellulose to other cell wall components[1]

and separate group of plant polysaccharides.[9]

The use of the term hemicellulose has changed frequently. Some investigators wish to confine the term to the non-cellulosic carbohydrates and to define hemicellulose as being composed of (1) pentosans, chiefly xylan and araban, yielding on hydrolysis the pentoses, xylose and arabinose; (2) hexosans, chiefly mannan and galactan; capable of being hydrolysed to the hexoses, mannose and galactose; and (3) polyuronides, mostly in the form of polyuronic acids. The wood and cellulose chemists have been accustomed to define the term hemicellulose as that portion of a plant material which is soluble in cold alkali solution.

In general, the name hemicullulose is restricted to land plants and is applied to the group of carbohydrates found in the cell walls of plants in association with lignin as an amorphous phase enveloping the cellulose strands and which can be extracted from plant material with dilute alkali, either hot or cold.[10] A wide range of molecular sizes and molecular shapes exist among hemicelluloses. Because of these differences and differences in their acidic properties, the polysaccharides which constitute the hemicelluloses vary among themselves in solubility. As a plant tissue ages, the solubilities of the polysaccharides decrease to some extent. Decreased solubility is most pronounced when the tissue material is dried. Insolubilization is not inherent in the drying process itself because it is possible to

dry plant material in such a way as to prevent loss of solubility or chemical reactivity,

The isolation of the cell wall carbohydrates is based upon their differences in solubility. The extent to which a soluble polysaccharide is extracted from a plant tissue or holocellulose depends upon the ease with which the solvent penetrates the insoluble phase and the facility with which the soluble component can diffuse from the matrix to the surrounding solution.

1.2 Molecular Structure of Hemicellulose

The group of polysaccharides called hemicelluloses are usually mixtures which include some or all of the xylans, the glucomannans and the arabogalactans. Sometimes other plant polysaccharides such as the β -glucans of barley and oats, some mannans and some galacto-mannans are regarded as hemicelluloses.

(a) D-Xylans

Xylans are a group of polysaccharides, having backbone chains of (1 - 4)-linked β-D-xylopyranosyl residues occur in all land plants[12] and in almost all parts of the plant.[13] Purified hemicellulose is, in many instances, identical to xylan.[14] Xylan occurs also in some marine algae. It is most abundant in annual crops, particularly in agricultural residues such as corn cobs, corn stalks, green hulls and stems where it occurs in amounts ranging from 15 to 30%. Hard woods contain 20 to 25% and soft wood contain 7 to 12% of xylan

TABLE I Chemical composition[a] of six North-American species of wood[10]

Species	Cellulose	Lignin	O-Acetyl	Hexuronic acid[b]	Residues[c] of				
					Galactose	Glucose	Mannose	Arabinose	Xylose
Acer rubrum Red maple	44.1	24.0	3.8	3.5	0.6	46.6	3.5	0.5	17.3
Betula papyrifera White birch	41.0	18.9	4.4	4.6	0.6	44.7	1.5	0.5	24.6
Fagus grandifolia American beech	42.1	22.1	3.9	4.8	1.2	47.5	2.1	0.5	17.5
Abies balsamea Balsam fir	44.8	29.4	1.5	3.4	1.0	46.8	12.4	0.5	4.8
Picea glauca White spruce	44.8	27.1	1.3	3.6	1.2	46.5	11.6	1.6	6.8
Pinus banksiana Jack pine	41.6	28.6	1.2	3.9	1.4	45.6	10.6	1.4	7.1

[a] All values in per cent of extractive-free wood

[b] As $C_6H_{10}O_7$ minus H_2O

[c] As $C_6H_{12}O_6$ minus H_2O

TABLE II Carbohydrate composition* of fifteen species of European Hardwood[11]

Species	Residues of:				
	Galactose	Glucose	Mannose	Arabinose	Xylose
Acer platanoides Norway maple	2.0	60.5	4.0	1.0	32.5
Alnus glutinosa Black alder	2.5	73.5	3.5	1.0	19.5
Alnus rugosa Speckled alder	3.5	67.0	1.5	1.0	27.0
Betula pubescens Birch	1.0	55.0	2.5	2.5	39.0
Betula verrucosa Silver birch	1.5	58.5	0.5	0.5	39.0
Corylus avellana European hazel	2.0	69.5	2.0	2.0	24.5
Fagus sylvatica European beech	4.0	65.0	1.5	1.5	28.0
Fraxinus excelsior European ash	3.0	60.0	2.5	2.5	32.0
Populus balsamifera Balsam poplar	3.5	68.0	2.5	2.5	23.5
Populus tremula European trembling aspen	1.5	64.5	3.0	1.0	30.0
Prunus padus European cherry	2.5	65.5	2.5	1.0	28.5
Quercus robur English Oak	2.5	68.5	2.0	1.0	26.0
Salix alba White willow	3.0	74.0	2.5	1.0	19.5
Sorbus aucuparia European mountain ash	1.5	66.5	2.5	2.0	27.5
Tilia cordata Linden	1.5	58.5	3.5	2.0	34.5

*All values in per cent of total neutral carbohydrates.

TABLE III Percentage composition of polysaccharides [21]

Source	D Glucuronic acid	D Galactose	L-Arabinose	Other	Reference
Acacia cyanophylla	24	49	7	L-Rhamnose 20	34
Acacia Karroo (Hayne)	12	50	36	L-Rhamnose	35
Acacia mollissima (Black wattle	9	42	42	L-Rhamnose 7	36
Acacia pycnantha	5	65	27	L-Rhamnose 1-2	37
Acacia verek (arabic)	16	52	19	L-Rhamnose 14	38
Amygdalus (Almond (tree)	10	30	40	D-Xylose 20	39
Anogeissus latifolia wall (Ghatti)	12	27	41	D-Mannose 8	40
Asparagus adscendens	10			D-Glucose 40, D-Mannose 40, D-Xylose 10	
Asparagus filicinus	5			D-Mannose 45, D-Glucose 40, D-fructose 10	
Boswellia carterii (olibanum or frankincense)		58	8	4-O-Methyl-D-glucuronic acid 33 trace of L-fucose and L-rhamnose L-Rhamnose 25	41
Brasenia schreberi Gmel (watershield)	22	47	9	D-Mannose 16	
Cetraria islandica	3	8		D-Glucose 89, D-Mannose 3	42
Citrus limonia (Lemon)	22	55	22	4-O-methyl-D-glucuronic acid	43
Citrus maxima	31	53	16	4-O-methyl-D-glucuronic acid	
Combretum verticillatum		15	38		44
Commiphora myrrha (Myrrh)		30	12	4-O-methyl-D-glucuronic acid 38	45
Curculigo orchioides	42			D-Mannose 23, D-glucose 35	
Dilsea edulis	10	80		D-xylose 7	46
Fagara xanthoyloides		61	21	4-O-methyl-D-glucuronic acid 17	47
Ferula species (Asafetida)	11	55	33	L-Rhamnose	48
Hakea acicularis	8	58		D-xylose 8, D-mannose 7	49
Hevea brasiliensis	6	4	20	D-Glucose 60, L-rhamnose 2, D-xylose 8	
Mimosa pudica	21			D-xylose 79, trace of D-glucose	
Moringa ptorygosperma (Drum stick)	10	37	53	Trace of L-rhamnose	51
Prosopis juliflora (Mesquite gum)		31	51	4-O-methyl-D-glucuronic acid 18	52
Prunus armeniaca (Apricot)	16	44	41		
Prunus cerasus (Cherry)	12	21	55	D-mannose 10, trace of L-rhamnose and D-xylose	53
Prunus domestica (Egg plum	15	40	34	D-xylose 11	46
Prunus insits (Damson)	16	30	38	D-mannose 15, trace of D-xylose	54
Prunus persica (Peach tree)	7	36	43	D-xylose 14, trace of L-rhamnose	55
Prunus serrulata	6-8			D-xylose 92-94	
Prunus species (Purple plum)	12	38	38	D-xylose 12	39
Prunus virginiana (Chokecherry)	9	26	29	D-mannose 13, L-rhamnose 23	37
Puya chilensis (Chagual)	15	36	7	D-xylose	49
Ulva lactuca (Green marine algae)	19			D-xylose 9, L-rhamnose 31, D-Glucose 8	56
Zea hays (Cornhull)	7-12	7	35	D-xylose 48	57

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TABLE IV Xylans from the gramineae

Source	Mode of linkage of L-arabinofuranose end-groups[a]	Mode of linkage of D-glucuronic acid end-groups[b]	Other structural features	References
Esparto grass	none present	none present		58
Esparto grass	(1-3)-D-Xylp	none present	β -D-xylp-(1-2)-L-Ara	59
Wheat straw	(1-3)-D-xylp	(1-3)-D-xylp (Me)	branched	50
			Xylan chain	60
Wheat straw	(1-3)-D-xylp	(1-2)-D-xylp	.......4-D-Gp-1......	61
Wheat straw	(1-3)-D-xylp	none present	3 ⋮	
Wheat straw	none present	(1-2)-D-xylp		
Wheat straw	(1-3)-D-xylp	(1-2)-D-xylp (Me)	some branched	62
			xylan chains	63
Oat straw	(1-3)-D-xylp	(1-2)-D-xylp (Me)		64
Cocksfoot grass	(1-3)-D-xylp	(1-2)-D-xylp (Me)		
Wheat leaf	(1-3)-D-xylp	(?)(1-3)-D-xylp		65
Barley husks	(1-3)-D-xylp	(1-2)-D-xylp (Me)	β -D-xylp-(1-2)-L-Araf	
			branched	
			xylan chain	
Corn (maize) cobs	(1-3)-D-xylp	not known	......2-L-Araf-1......	16
Corn (maize) cobs	(1-3)-D-xylp	(1-2)-D-xylp (Me)	β -D-xylp-(1-2)-L-Araf	16
		(1-4)-D-xylp		66
Corn (maize) cobs	(1-3)-D-xylp	none present	......4-D-Gp-1...... 3 ⋮	61
Maize fibre	(1-3)-D-xylp	not known	α -D-xylp (1-3)-L-Ara	67
			L-Galp-(1-4)-D-xylp-(1-2)-L-Ara	
Maize hulls	not known	(1-2)-D-xylp	α -D-xylp (1-3)-L-Ara	68
			β -D-Galp-(1-4)-D-xylp	69
			β -D-Galp-(1-5)-L-Araf	
Wheat bran	(1-3)-D-xylp	(1-2)-D-xylp	......3-L-Araf-1......+	70
	(1-3)-D-xylp-(2-1)			18
Wheat flour	(1-3)-D-xylp	none present		
	(1-3)-D-xylp-(2-1)			
Barley flour	(1-3)-D-xylp	none present		71
	(1-2)-D-xylp			
	(1-3)-D-xylp (2-1)			72
Rye flour	(1-3)-D-xylp	none present		

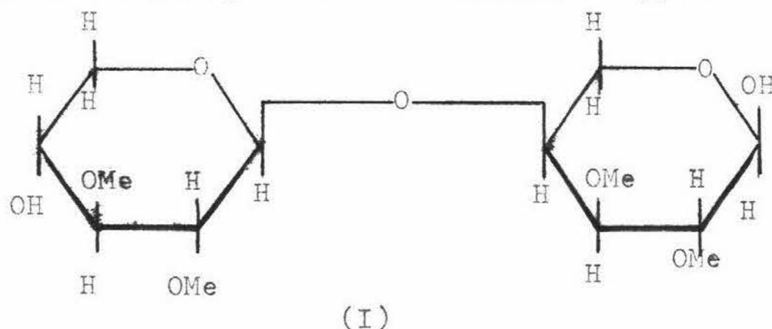
[a] D-Xylp = D-xylopyranose, L-Araf = L-arabinofuranose, D-Gp = D-glucopyranose and Galp = galacto-pyranose residues

[b] (Me) indicates that D-glucuronic acid residues are present, wholly or in part, as the 4-methylether

TABLE V Composition of the linear A and B and the branched B polymers from some gramineae and leguminosae [87]

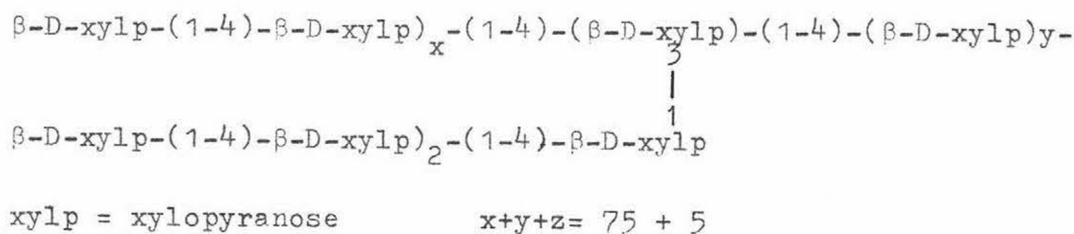
	T.pratense (red clover) (%)	M.sativa (lucerne) (%)	G.max (soya bean) (%)	L.perenne (grass) (%)	T.vulgare (wheat) (%)	Z.mais (maize) (%)
Linear A						
uronic acid	4.7	6.6	6.6	1.9	2.1	2.4
galactose	-	-	-	-	-	-
arabinose	-	-	-	12.9	5.7	5.3
xylose	95.3	93.4	93.4	85.2	92.2	92.3
glucose	-	-	-	-	-	-
Linear B						
uronic acid	1.0	1.5	4.3	0.4	0.3	0.1
galactose	-	-	-	-	-	-
arabinose	10.8	8.3	8.3	16.5	11.1	10.9
xylose	88.2	90.2	89.2	83.1	88.6	89.0
glucose	(11.6)	(11.4)	(4.0)	(11.0)	(12.0)	(21.5)
Branched B						
uronic acid	20.6	22.3	24.6	5.4	7.9	12.8
galactose	34.5	31.1	34.3	7.7	9.8	8.7
arabinose	27.6	34.2	24.0	23.8	26.5	24.4
xylose	17.3	3.1	3.4	63.1	55.8	54.1
glucose	-	9.3	6.9	-	-	-
rhamnose	-	-	6.8	-	-	-

(see Tables I, II and III). Low strength vegetable fibres of commerce such as jute, sisal, manila hemp and coir may contain 5 to 20% xylan, whereas high strength fibres such as ramie, flax and cotton are almost devoid of xylan.[15] The xylan of esparto grass is of particular interest, for it is a true D-xylan, composed exclusively of D-xylose residues.[16] Hydrolysis of esparto xylan gives D-xylose in 95 to 98% yield. Further hydrolysis of the methylated xylan produces 2,3-di-O-methyl-D-xylose (92%), 2,3,4-tri-O-methyl-D-xylose (2.6%), 2-O-methyl-D-xylose (5%) and a trace of 2,3,5, tri-O-methyl-L-arabinose. Acetolysis of the methylated D-xylan gave the disaccharide (I), therefore the glycosidic linkage is (1-4) and the D-xylose residues are in the pyranose form.[17]



(I)

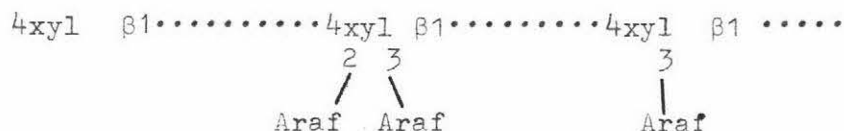
From this evidence the main features have been formulated as



The presence of chains of (1-4)β linked xylopyranose residues appears to be a general structural feature throughout most of the xylan group of polysaccharide (see Table IV).

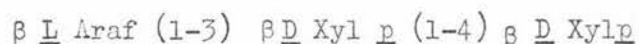
(b) Arabino-Xylans

Perlin[18] has isolated from wheat flour a xylan fraction which contains almost as much arabinose as xylose. Investigation of this arabinoxylan by the methylation procedure and by other methods has shown that single L-arabofuranose residues are attached to a main chain of xylose residue, in many instances substituting the hydroxyl at both C-2 and C-3 of the same xylose residue. These arabofuranose residues were readily removed by acid hydrolysis, leaving a xylan similar to the arabinose free material from esparto grass. The general molecular structure of wheat flour xylan is



However, some xylans, such as the hemicelluloses from corn (maize) cobs, maize fibre and maize hulls and barley husks also contain non-terminal L-arabinofuranose. Such L-arabinofuranosyl linkages are easily released under mild conditions of hydrolysis, therefore oligosaccharides produced from them have L-arabinose residues at the reducing end.[19]

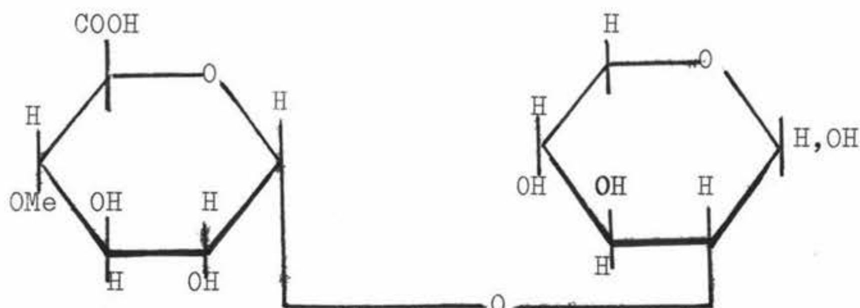
Bishop and Whitaker[20] have isolated an enzyme from myrothecium verrucaria which hydrolyses linear chains of β-(1-4) linked D-xylose units. Application of the enzyme to wheat straw hemicellulose yields among other products, L-arabinofuranosyl - (1-3) β-D-xylo-pyranosyl - (1-4) D-xylose.



The isolation of this trisaccharide confirms the mode of attachment of L-arabofuranose residues to a xylan unit in the main chain.

(c) Glucurono-xylans

Many polysaccharides of the xylan group contain residues of D-glucuronic acid or 4-O-methyl-D-glucuronic acid.[21] As glycosiduronic acids are very resistant to mineral acid hydrolysis, aldobiouronic acids may be isolated from the graded acid hydrolysis of xylan containing hexuronic acid residues and the mode of linkage of D-glucuronic acid to D-xylose may be determined by the identification of the hydrolysis products of the derived methylated aldobiouronic acid. Jones and Wise[22] have shown that O-(4-O-methyl -α-D-glucosyluronic acid) (1-2)-D-xylose can be isolated from aspen wood hemicellulose.



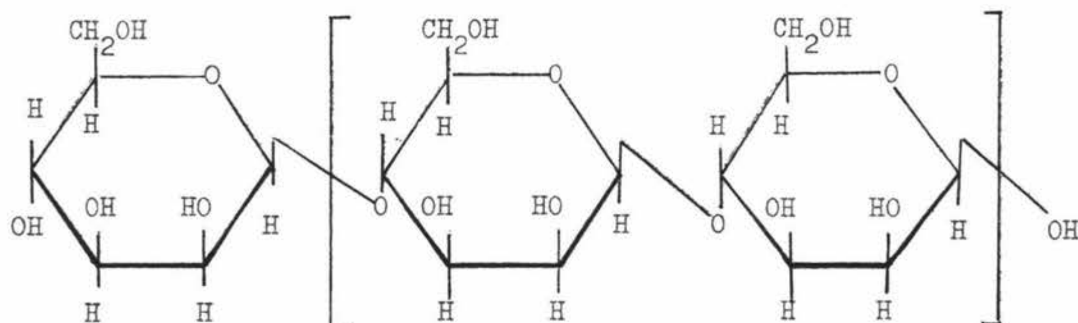
D-glucuronic acid or its 4-methyl ether are most commonly linked to position 2 of D-xylose, although similar aldobiouronic acids containing (1-3) wheat straw) and (1-4) linkages (corn cob) have also been observed.

In the structural study of hemicellulose of Lucerne, Aspinall and McGrath[23] have found that the polysaccharide contains an essentially linear chain of 1-4 linked β-D-xylopyranose redisues with side chains of 4-O-methyl-α-D-glucopyranosyluronic acid residues attached to C-2 of approximately every ninth residue. The main chain also contains occasional rhamnose residues.

(D) Mannans

Polysaccharides composed almost entirely of D-mannose residues are the chief constituents of palm seed endosperm, occurring as food reserves that disappear on germination. They are also the major structural unit occur in woods and in the seed of many plants. Two morphologically distinct D-mannans are isolated from vegetable ivory. D-Mannan A is extracted with alkaline solution and gives crystalline x-ray patterns in the native state and when precipitated from alkaline solution.[24] D-Mannan B occurs as microfibrils analogous to those of cellulose. It has a higher molecular weight than D-Mannan A and is not readily soluble in alkaline solutions, but it is soluble in cupra-ammonium hydroxide solution. On precipitation it gives an amorphous x-ray pattern.[24] Methylation and hydrolysis of these polysaccharides yields 2,3,6-tri-O-methyl-D-mannose as the main product,[25] while partial acetolysis followed by deacetylation yields a β-D-(1-4) - linked disaccharide (mannobiose), mannotriose,

and higher homologs.[26] Thus the D-mannans must be linear chains of D-mannopyranose residues linked by β- (1-4)-glycosidic bonds (II). D-Mannans have been defined[27] as polysaccharides containing 95% or more of D-mannose residues.



(II)

In addition to their occurrence in ivory nuts, they are found in green coffee beans[28] in tubers of various species of orchids (salep mannan),[29] and in the seaweed alga Porphyra umbilicals. All have the same general chemical structure,[30] but they appear to differ in chain length. The glucomannans which comprise up to half the hemicellulose content of some coniferous woods contain units of both mannose and glucose in their molecular structure.[31] Many mannans and glucomannans contain a small proportion of D-galactose residues, which have been shown to be an integral part of the mannans or glucomannans.

(e) <u>Arabino-galactans</u>

L-arabino-D-galactans are the major wood glycans that can be extracted from wood with water before delignification. They are water-soluble, highly branched and are found in the wood of conifers. Their ease of extraction and their useful qualities as gums have brough them into commercial production marketed as

the commercial gum, Stractan, Polysaccharides of this type have (1-6) and (1-3) linked D-galactopyranose residues and the L-arabinose residues appear as integral parts of these arabino-galactans.

Jones and co-workers[32] have found that the addition of borate to the alkali permits the extraction of the glucomannan in reasonably pureform as the borate complexes. By controlled acid hydrolysis of Loblolly pine wood, Jones and Painter[33] have isolated 2-O-(4-O-methyl-α-D-glucoronosyl)-D-xylose and 4-O-β-D-glucopyranosyl-α-D-mannopyranose.

The greater part of cell wall material in wood, other than cellulose itself, are the xylans, (including arabinoxylans and glucuronoxylans) the galactoarabans and the glucomannans.

1.3 Preservation, Isolation and Extraction of Hemicelluloses from Tall Fescue (*Festuca arundinacea*)

(A) The Plant Material

Tall Fescue (*Festuca arundinacea*), a perenial grass was grown by the Grasslands Division of the Department of Scientific and Industrial Research. The grass was cut in April and was immediately frozen to prevent chemical changes.

(B) Drying and Storage of grass

To arrest chemical changes the grass must be frozen in the

deep freeze as soon as possible after cutting. There are several methods for preservation of grass. Melvin and Simpson used forced air drying at 21 °C but this causes extensive respiratory losses of carbohydrates and also non-volatile organic acids.[73] Hirst and Ramstad[74] found that a forced hot air draught at 70 °C caused smaller losses than the previous method. Davies, Evans and Evans[75] introduced freeze-drying method and this has been found to be less damaging than oven-drying.[76,77]

The changes in carbohydrates and amino-acids during the storage of freeze-dried herbage have been studied.[78] Since some enzymes can function in the presence of the residual 5 - 10% of water in freeze-dried tissues, it is advisable to analyse samples for readily respired constituents, such as soluble carbohydrates, amino acids and organic acids, as soon as possible. With precautions such as storage under nitrogen, at low relative humidity and at temperatures as low as - 20 °C it is possible to minimise these chemical changes. Czerkawski[79] found no difference in the cellulose and lignin contents of grasses dried at 50 °C and 100 °C but noted a considerable increase in these constituents when samples were stored at relative humidity as high as 80%.

The conclusion of most investigators is that there is no universal method of drying plant tissue. No method can be relied on for consistent results, since the appropriate drying

temperature depends upon the chemical and physical composition and enzyme content of the plant.

In this investigation, freeze - drying was used, primarily to preserve the nonvolatile organic acids intact. Extractions were then carried out within two weeks of drying. The sample was cut with hand-shears and immediately frozen in solid carbon dioxide prior to freeze-drying. Any dead leaves were removed as the frozen leaves were being spread on the freeze-dryer tray. The material was freeze-dried and the dry plant material then ground in a Wiley mill to pass a 1 mm sieve and stored under vacuum with silica gel.

(C) Extraction and Purification Procedure

The extraction scheme for the fractionation of polysaccharides from the dry grass is outlined in Fig. II. This exhaustive procedure, using mild extractants, was used to isolate the structural polymers with the minimum of modification or degradation. In order to avoid oxidation and the onset of "horniness", the residues after each extraction step were not air-dried, except for the initial benzene/ethanol extract.

(D) Preliminary Extraction

The dried and powdered grass is first extracted with an azeotropic mixture of benzene/ethanol (2:1 V/V) and then the

residue extracted with warm water. Lipids, chlorophyll, organic acids and some other extractives are removed in the first extraction while water-soluble polysaccharides and fructosans are removed later. The removal of lipids and extractives before polysaccharide separation not only eliminates them as sources of impurities but opens the tissues to penetrate by hydrophilic solvents.

The choice of 60°C for the temperature of water for extraction followed the practice of earlier work concerned with the extraction of fructosan from dried grass.[80] As the temperature increased, the amount of water-soluble polysaccharide being extracted also increased. At 60°C, the extract came largely from the ionic fraction, and at the temperature higher than 60°C, an appreciable amount of extract came from non-ionic fraction. Therefore, the water at 60°C is the best condition for extracting water-soluble polysaccharides.

(E) Pectic substances

The pectic substances consist of pectin together with galactan and araban. They are found abundantly in the primary cell walls so that young plants may be rich in them. Mature plant material contains only small amounts and the pectic material is often associated with polymers of glucose and even of xylose. The method of Weilhe and Phillips[81] using 0.5%

ammonium oxalate at 80-85°C for twenty-four hours can be used to remove the pectic substances, but has been shown that even after thorough washing with hot water, there is still ammonia left in the residue. Solutions of oxalic acid, ammonium citrate, fluorides, arsenates and phosphates have been employed, but pectic substances such as polygalacturonic acids are better extracted by 2% (W/V) solution of EDTA (sodium salt at pH 6.7) at 70°C.[23]

(F) Deproteinisation

After those three previous extractions, the grass residue still contains a large amount of the original nitrogen and most of the protein. As an examination of the structural carbohydrates is a major object of this work, reagents to be employed in the removal of protein have to be mild enough not to bring into solution any appreciable quantity of the hemicellulose fraction. This precluded the use of alkali, therefore the proteolytic enzyme pepsin was used. This has been shown to have no activity towards carbohydrates.

(G) Delignification

Lignin is a complex, three dimensional polymer of phenylpropane residues, formed by dehydrogenation polymerisation of a precursor which is probably of the coniferyl alcohol type.[83] Lignin removal from the material to be used in structural investigation requires more than usual care because of the ease

with which the water soluble hemicellulose fraction goes into solution once a substantial amount of the lignin is removed.[82] However, lignin must be removed as it often retards or prevents complete extraction of the hemicelluloses, either because of machanical obstruction or by reason of attachment through covalent bonds. Bouveng and Lindberg[84] have observed that hemicelluloses containing lignin could not be separated into the pure components, but, after further delignification, they could be readily fractionated. Delignification may cause a loss in carbohydrate, therefore as a mild treatment as possible is desirable. Delignifi-cation with chloramine in weakly acid solution was suggested by Gaillard[2] who found that the treatment compares favourably with the usual acid chlorite treatment. For plant material with a moderate lignin content (6-11%) such as hay and fresh grass, deligmification can be carried out directly after the extraction with EDTA or ammonium oxalate solution without any loss in carbo-hydrates. After the suspension was filtered and washed with ethanol, solution of 3% ehtanolamine[2] was left in contact with the residue. The purpose of this is to remove secondary cell wall lignin without attacking that of the middle lamella.

(H) <u>Hemicellulose Extraction</u>

The hemicelluloses are a mixture of polysaccharides of different solubility, part being readily soluble in weak alkali and the remainder being extracted only by strong alkaline solution.

Alkaline extraction of holocellulose is a useful way of isolation a group of polymers which can later be separated. However, alkaline extraction of the holocellulose can bring many changes in the polysaccharides, even under oxygen free conditions, alkaline degradation might result. It is also possible that any naturally occurring ester groups are saponified.[1]

Solution of potassium hydroxide, instead of sodium hydroxide are often used for the extractions because of the high solubility of potassium acetate in ethanol. The alkaline extraction is usually carried out at or below room temperature for limited periods of time and in an atmosphere of nitrogen. Under these conditions, any changes in the xylan brought about by the alkali are kept at a minimum.

The subdivision of the hemicelluloses into individual molecular species has not been accomplished. Separation difficulties arise not only from the presence of different kinds of molecules, but also from the occurrence of each molecular type in different degrees of polymerisation. Therefore, separation based on solubility would not bring about a complete segregation of species. A good general method for effecting initial subdivision of the hemicellulose group is the neutralisation of an alkaline hemicellulose solution which causes precipitation of the high molecular weight polymers and leaves in solution the molecules of lower degree of polymerisation.

The more soluble fraction is often called polyuronide hemicellulose or hemicellulose B and the less soluble xylan cellulosan or hemicellulose A.[85] The difference in solubility of these polysaccharides depends mostly upon the chain length and upon the presence of a uronic acid unit in the chain. Molecules with shorter chain lengths or those containing uronic acid units may have a higher solubility but otherwise the nature of the sugar units has little effect on solubilities.

Hemicellulose A was precipitated by acidifying the extract to pH 5.0 by adding 50% aqueous acetic acid. The suspension was centrifuged and hemicellulose B precipitated by pouring the clear filtrate into ethanol.[86] The precipitate (pale brown in colour) was collected on a nylon gauze and dissolved in water to give a 4% solution. Not all the precipitate would redissolve in water, so the water-insoluble fraction (hemicellulose B (I)) was centrifuged off. The aqueous hemicellulose B solution was centrifuged and poured into five volumes of 95% ethanol. The precipitated hemicellulose B was a white, porous compound after frozen and freeze-dried while hemicellulose B (I) was pale brown and was probably contaminated with lignin.

(I) Fractionation of Hemicellulose B

Hemicellulose B is a mixture of several different polymers, both linear and branched. The separation of these individual

polymers in a pure state is a tedious procedure. Generally it is achieved by repeated fractional precipitation from aqueous solution with ethanol and acetone. Gaillard[87] observed that it is possible to separate the linear from the branched polymers in hemicellulose B by dissolving the mixture in concentrated calcium chloride solution and precipitating the linear polymers with an iodine-potassium iodide solution. The branched polymers are then recovered from the filtrate.

1.4 Homogeneity of the Polysaccharide[88,89]

Most natural occurring polysaccharides are complex mixtures. So that for the complete structural characterisation of a polysaccharide, it is necessary to ensure that the polymer is pure, has been isolated from a single species and is chemically homogeneous. This means that further attempts at fractionation of the polysaccharide do not change the specific rotation and the ratio of mono saccharide building units. There is no standard method for the isolation of a polysaccharide from a mixture, a combination of several methods usually gives the best chance of success. Separation and purification are often difficult and tedious, but is an essential preliminary to structural determination.

As extracted polysaccharides are usually mixtures contaminated by other substances such as protein and lignin. The extraction procedures may modify the actual structure of the molecule and also alter its molecular weight distribution. The ease of

purification depends upon the nature of the contaminating substances.

This is true of the hemicelluloses which can be differentiated in terms of 'polymolecularity', 'polydispersity' and 'polydiversity'. The methods used for the separation of hemicellulose mixtures into homogeneous polysaccharides include graded extraction, copper complex formation[91], ethanol precipitation[92,93] or by cooling, quaternary amononium salt formation,[94] ultra-centrifugation, electrophoresis, chromatographic adsorption, gel filtration, the use of enzymes and immunological tests. The homogeneity after separation can be determined by ultra-centrifugation,[95] free-boundary electrophoresis,[96,97] paper ionophoresis[98,99] or high voltage zone electrophoresis.[100,101]

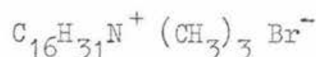
Fractionation of Polysaccharides

While electrophoresis and ultracentrifugation are excellent methods for fractionating polysaccharide they are usually only applicable to the separation of microquantities.

It is more common to use fractional precipitation. A solution of the precipitant is added to an aqueous solution of the polymers. However separation may be poor due to the tendency towards coprecipitation and occlusion of other polysaccharides.

(i) Jones[102] found that cetyl trimethylammonium bromide (III) could be used to precipitate the acidic components from a mixture of polysaccharides and Cetylpyridinium bromide (IV) is also the most

commonly used.



(III) (IV)

(ii) Fehling's solution is often employed to selectively precipitate polysaccharides. The galacto-and glucomannans are precipitated by all reagents. Galactans with 1,4-β-linked galactose residues and some uronic acid residues (no cis-glycol) is not precipitated with Fehling's solution. Solutions of barium hydroxide, basic lead acetate, lead acetate cupriethylene diamine, copper chloride, copper sulphate and copper acetate have also been used.

1.4.1 Determination of the Homogeneity of Polysaccharide

The methods which can be used are:

(a) Fractional precipitation with ethanol
(b) Precipitation using a specific complexing agent.
(c) Electrophoresis

(a) Fractional Precipitation with Ethanol

This involves the precipitation of polysaccharide from solution by the gradual addition of ethanol to the aqueous solution of water-soluble polysaccharide.

The fractionation is usually carried out at or near pH 7.0, where the polysaccharides are most stable, and the carboxyl groups, present in the hemicelluloses containing uronic acids, are in the form of ionised

salts. Under acidic conditions, significant hydrolysis of glycosidic bonds may occur and in basic solutions, alkaline degradation may take place.

If the polysacchardie is homogenous it precipitate as a single peak over a relatively narrow ethanol concentration.

(b) Precipitation using a Specific Complexing Agent

Copper salts have been widely used as precipitating agents to tractionate mixtures of polysaccharides. Fehling's solution is often used possibly because it is a common reagent in the laboratory.

Usually an excess of precipitant is added, but a large excess of Fehling's solution should be avoided since the precipitated "copper complex" is sometimes soluble in excess reagent. The insoluble polysaccharide - copper complex is removed by either filtration or centrifugation. The jelly-like complex is then decomposed by an alcoholic solution of acid or a chelating agent and the precipitate is washed until free from inorganic ions.

(c) Electrophoresis

This involves paper ionophoresis , free-boundary electrophoresis or high voltage zone electrophoresis. Frahn and Mills[98] used four electrolytes in paper ionophoresis of carbohydrates. The found that sodium

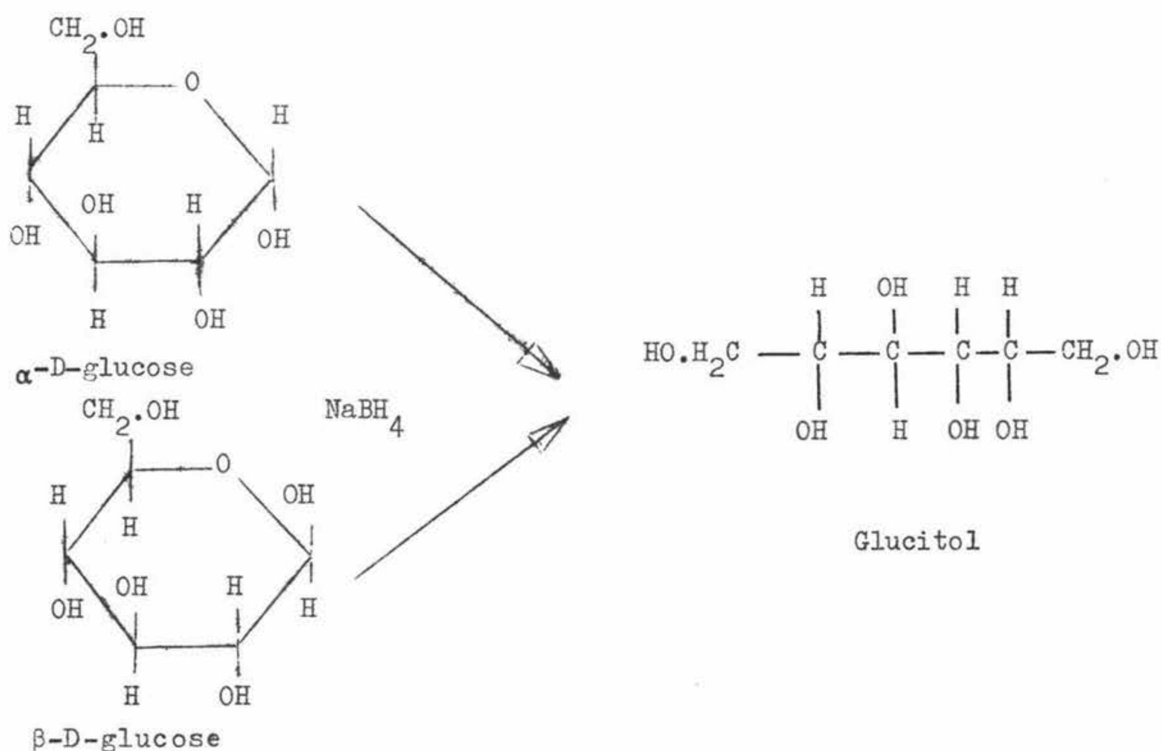
arsenite and basic lead acetate are the most effective electrolytes for separating reducing sugars, basic lead acetate is the most effective for separating sugar alcohols, while borax is the best for simple glycols. Dudman and Bishop[101] also found that electrophoresis of dyed polysaccharides can be done on cellulose acetate paper using 0.1 M sodium tetraborate-sodium chloride buffer as electrolyte. The dyed polysaccharides give visible bands on cellulose acetate strips. Separation of polysaccharides by this method are complete within five minutes and match the results obtained with the undyed polysaccharides by free-boundary Tiselius electrophoresis.

1.5 Quantitative and Qualitative Determination of Sugars by Gas-Liquid Chromatography[103,104,105,106]

Gas-liquid chromatography is a valuable supplement to existing analytical methods, because of its greater selectivity, speed and simplicity. As a result it has been widely used for the separation and estimation of carbonhydrates and related polyhydroxyl compounds. Since these substances are not themselves sufficiently volatile for gas chromatography, suitably volatile derivatives have to be made, and these are commonly prepared from the sugars by methylation or acetylation.

The work of Sweeley, Bentley, Makita and Wells[107, 108] is one of the most significant advances in carbohydrate chemistry in the decade. Using a simple experimental procedure[109] they prepared the polytrimethylsilyl ethers of sugars and chromatographed these derivatives on several different supports with spectacular qualitative results.

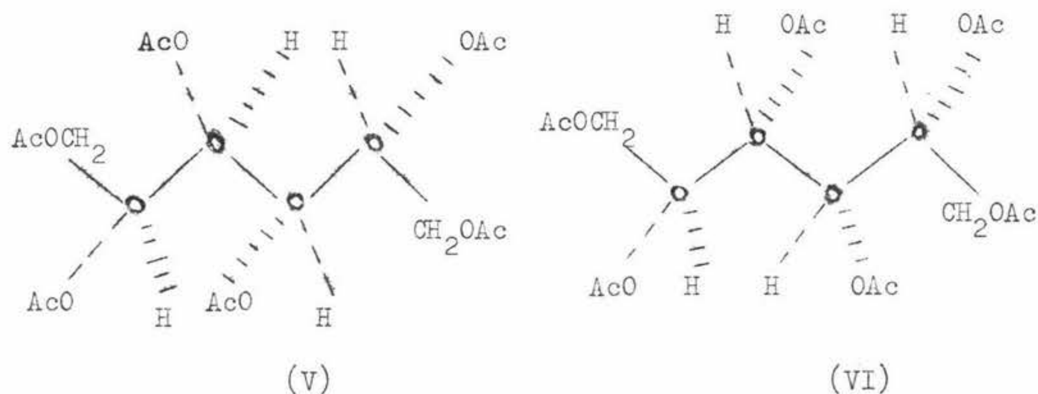
The sugars can be converted into their derivatives quantitatively, but problems arise when separating monosaccharides by gas liquid chromatography. Anomerisation and ring isomerisation can result in the formation of as many as four glycosides from each monosaccharide. Each of these glycosides will produce a peak on the chromatogram. In a complex sugar mixture, containing a number of monosaccharides, the multiplicity of peaks produced would prevent complete separation of all the peaks from one another, as a result, accurate quantitative determination cannot be achieved. However, this difficulty can be overcome by converting the monosaccharides into their corresponding alditols, by reduction with sodium borohydride and subsequent acetylation.[110] This procedure eliminates the problem of multiple peaks since the alditols cannot anomerise,[111] and the method is applicable to the quantitative analysis of the sugars in hemicellulose B.



Initially trimethylsilyl derivatives were formed by the method of Sweeley, Bentley, Makita and Wells[107] but, as in their investigation, it was found that arabitol/xylitol and galactitol/glucitol mixtures could not be separated on an SE-52 column (Silicone gum rubber, phenyl). Consequently, alditol mixtures were acetylated with a mixture of pyridine/acetic anhydride 1:1 ; V/V (4ml) in a boiling water bath for 12 minutes.[112] The mixture was evaporated under reduced pressure to a syrup which was dissolved in ethyl acetate (2ml) for injection into the gas chromatograph.

It has been claimed[113] that a relationship exists between the retention times of the alditol acetates and their stereochemical structure as described in the preferred zigzag-conformations. For alditol acetates of the same molecular weight, the greater, the number of acetoxy groups or non-terminal carbon atoms which are arranged on one side of the molecule, the greater the affinity of the compound for the liquid phase and hence the longer its retention time. Furthermore, it has been postulated that for alditol acetates of the same molecular weight and the same number of acetoxy groups arranged on one side of the molecule, the closer these acetoxy groups are to each other the greater is the affinity of the compound for the liquid phase and hence the greater the retention time. These empirical rules also predict the present difficulty of separating glucitol- and galactitol hexa -acetates.

Galactitol hexa-acetate (V) in the zig-zag conformation has two acetoxy groups in close proximity on either side of the molecule while in glucitol hexa-acetate (VI) there are three consecutive acetoxy groups on one side of the molecule.



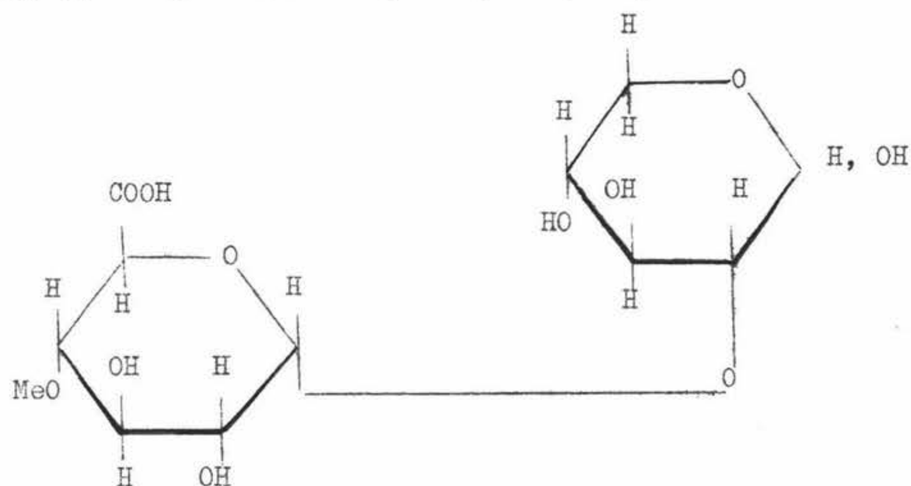
On the basis of these 'rules' of Gunner et al, it is reasonable to expect the affinity of each acetylated molecule for the liquid phase to be roughly similar, hence their difficulty of resolution.

In the present analytical work, the hexitol hexa-acetate peak was designated 'glucose', 'galactose' or both, on the basis of paper chromatographic analysis of an aliquot of the original hydrolysate. However, since the peak area/weight ratios for both derivatives were so similar, it was possible to calculate the glucose plus galactose content of an unknown by treating the hexose peak as an entity.

1.6 Quantitative Determination of Uronic Acids[114, 115]

The uronic acids, 4-O-Me-α-D-glucopyranosyluronic acid

(woody plants) or α-D-glucopyranosyluronic acid (annual plants), are generally attached to C-2 or C-3 of the xylan chain of hemicelluloses.[9] This linkage is resistant to hydrolysis in sulphuric acid up to a strength of 0.5 M so that acid hydrolysis gives rise to an aldobiuronic acid, usually 2-O (4-O-Me-α-D-glucopyranosyluronic acid) -D-xylose. VII)



(VII)

Such aldobiuronic acids do not move from the origin with paper chromatography in the solvents used since their Rfs are lower than those of free uronic acids. Furthermore they do not form suitable derivatives for gas-chromatographic separations and are probably lost as the barium salts in the neutralisation of the hydrolysate.

Consequently, the uronic acid content of the hemicullulose and pectic (polygalacturonic acid) fractions was determined directly on the free polymers by the spectrophotometric carbazole method of Dische.[114]

The decomposition of polyuronides by hot hydrochloric acid with the formation of furfural and carbon dioxide is a common method for the determination of uronic acids. However it is not specific, as true sugars and amino sugars both give rise to the same products under the same conditions, and also it usually requires a few mg of material for several determinations.

Since 1929, Dische's method has been widely used for the microanalysis of uronic acids. Sample is treated with concentrated mineral acids (sulphuric or hydrochloric acid) to yield mixtures of products which react with various organic substances (indole, diphenylamine, carbazole and SH compounds) to give colours. Different groups of sugars such as pentoses, hexoses, deoxypentoses and even individual sugars of the same group (e.g. mannose and glucose) show marked differences in the speed of the development of the colour and in its absorption spectrum. The absorption due to the carbazole reaction of true sugars can be deducted when the nature of the sugar and its approximate concentration are known. Since hexoses, and to a lesser extent pentoses contribute to the colour formation, blanks are used containing the proportions of xylose, arabinose, glucose and galactose in the hemicellulose. Sample absorbences were measured at 530$m\mu$ in a Unicam SP 500 spectrophotometer and contained 10-100 μg uronic acid and a sugar concentration no greater than 0.02% W/V, a level which gave only a minor contribution to the total absorbence.

It should be noted, however, that the carbozole method does not give accurate determination of absolute hexuronic acid content since the colour intensity varies slighly with the type of polysaccharide investigated.[114] Nevertheless, the polysaccharide fractions studied were broadly similar in monosaccharide content and were free of interfering proteins and sulphydryl containing compounds so the method was valuable for determining the comparative uronic acid contents.

Meyer, Bloch and Chaffee[116] have reported that it is possible to determine the uronic acids in certain polyuronides after isolation and methanolysis by the naphthoresorcinol reaction. However, in the presence of proteins and true sugars the results are unsatisfactory.

Anthrone sulphuric acid and orcinol sulphuric reactions[117] are able to be used for identification of sugars, uronic acids and sugar amines, as they all give a colour reaction. The anthrone sulphuric reaction is better than orcinol sulphuric, as it has been reported that for equal quantities of glucuronic acid and galactose, the absorbance of glucuronic acid to galactose is 0.44 for the reaction with orcinol sulphuric acid, and 0.10 for the reaction with anthrone sulphuric acid. As it is impossible to obtain reproducible results with the same solution of sugars, because the absorbance varies, it is therefore necessary to introduce an internal standard into a series of determinations.

The simplicity, specificity of the carbazole and anthrone sulphuric acid reactions, and also the low degree of interferences from other constituents, encourage the application of these methods to quantitative determination of uronic acids in polysaccharides.

1.7 Paper Chromatography[51,118,119,120,121]

Since the introduction of paper partition chromatography in 1944, it has been successfully applied to the separation of sugars and also for their quantitative determination. The method gives a rapid means of separation as well as giving a strong indication of their identities. Though the final proof of their constitution depends upon their separation and identification by determination of their physical constants and the formation of characteristic derivatives.

1.8 Methylation Procedures: Which Include Methods of Methylation, Acid Hydrolysis, Qualitative and Quantitative Identification of the Monomers[84,122]

Methylation analysis is a very important method for structural investigation. The procedure involves the preparation of an exhaustively methylated polysaccharide followed by hydrolysis and identification of the resulting partially-O-methylated derivatives. The purpose of methylation is to acheive an etherification of all the free hydroxyl groups in the polysaccharide. Complete methylation can convert all unsubstituted

hydroxyl groups to methoxyl groups and each of free hydroxyl group present in the methylated monosaccharides that is liberated by hydrolysis corresponds to a substituted hydroxyl of the original product. Therefore, the glycosidic linkage and the ring size can be deduced.

The ideal methylating agent is one which can rapidly introduce the theoretical number of methyl groups and at the same time, cause no degradation of the sugar. However, it is very difficult to get these reagents with such properties, therefore, several methylations with different reagents are generally used in order to obtain full methylation.

The original, classical methods of Denham and Woodhouse[123] and Haworth[124] are still widely used. The polysaccharide is treated several times with 25-30% aqueous alkali and dimethyl sulphate. It has been reported that the use of more concentrated sodium hydroxide solution[125] and an increase of the operating temperature and even the replacement of sodium hydroxide by potassium hydroxide[126] give a more satisfactory result.

In general, polysaccharides are insuluble in the organic solvents, therefore they are usually methylated first as above. This yields a partially methylated product which is soluble in the methyl iodide required for the Purdie procedure. The method of Kuhn and his co-workers is also frequently used. The partially methylated product is treated with methyl iodide and

silver oxide under reflux. The advantage of this method is the ease with which the methylated product may be recovered. Other methods such as dissolving the polysaccharides in N,N-dimethylformamide [127] and then treating the solutions with methyl iodide or treating the polysaccharide in methyl sulphoxide with sodium hydroxide and methyl iodide[128] have also been used extensively.

It is very difficult to estimate the completeness of methylation, and the usual method is the determination of the methoxyl content and a comparison of this with a theoretical determination of the suspected repeating unit which is defined as the simplest repeating structure which defines the polysaccharide molecule.

The impurities, non-polysaccharide materials such as salts, condensation products and lignin and also part of the polysaccharide that is less completely methylated can be removed by fractional precipitation from the methylated polysaccharide. Mixtures of chloroform and light petroleum or ether are usually used for this purification.

Since the methylated polysaccharides are usually insoluble in water, a non-aqueous or only partially aqueous medium is used for the initial hydrolysis. Methanolysis is usually carried out by refluxing the methylated products with methanolic hydrogen chloride. Other methods include formolysis[129] and prehydrolysis[130] in concentrated sulphuric acid.

The aim of hydrolysis is to depolymerise the methylated polysaccharide to the monomeric state under conditions which give the least possible degradation of the resultant monomers. Because of the great resistance of glycosidic linkages to acid hydrolysis, uronic acid residues always appear as methylated aldobiouronic acids in the hydrolysate.

The mixtures of methylated sugars are separated by partition chromatography on a cellulose column, or by adsorption chromatography on carbon-celite columns. The components are tentatively identified on the basis of their mobilities on paper chromatography and electrophoresis. Since these products do not differentiate between D and L enantiomorphs, the components of the hydrolysate are then converted into appropriate derivatives, which can be identified by their melting points and optical rotations. Certain components of polysaccharides can be identified by specific colour reactions without previous direct hydrolysis and separation.[51]

Gas liquid chromatography is another important method in methylation analysis, both as an aid in identifying individual methylated sugars and for their quantitative measurement. It has been found that methyl ethers of the methyl glycosides of the heat labile monosaccharides are sufficiently stable and volatile to be analysed and the fully methylated methyl glycopyranosides are successfully separated by gas liquid partition chromatography.[131]

TABLE VI: CARBOHYDRATE FRACTIONS OF GRASSES AND CLOVERS[132]

Carbohdrate	Composition	Plant Function	Solubility
Mono-, di, tri-saccharides	Mainly glucose, fructose and sucrose	Soluble reserve	Water soluble
Fructosan	Fructose polymer		
Starch	Glucose polymer		Water dispersable
Pectin	Galacturonic acid polymer	Cell cementing substance	Cold dilute acid soluble
Hemicellulose	Complex mixture of polymers of xylose arabinose, glucose, galactose and uronic acid	Cell walls	Water insoluble; dilute alkali soluble
Cellulose	Glucose polymer		Water & dilute alkali insoluble

CHAPTER II

DISCUSSION

The polysaccharide under study was extracted from Tall Fescue (*Festuca arundinacea*) by extracting the grass with (i) boiling azeotropic ethanol-benzene (1:2) to inactivate enzymes and to remove soluble sugars, (ii) with warm water to remove the water-soluble polysaccharides, (iii) with ethyline-diamine tetraacetic acid disodium salt which removed the pectic materials and (iv) with pepsin in hydrochloric acid to remove protein. The residue was delignified with chloramine T and glacial acetic acid, and then it was further extracted with alkali to give a hemicellulose fraction. The hemicellulose B, dissolved in calcium chloride solution, was fractionated with iodine and potassium iodide solution into linear and branched polymer. The branched hemicellulose B was purified by forming a copper complex with Fehling's solution and further purified by fractional precipitation. In the fractional precipitation with ethanol (see section 3.2, p. 52) five fractions were collected which represented 86.4% recovery of the starting materials for hemicellulose B fraction B and 73.62% of the total polysaccharides precipitated in the narrow range of 50.1 to 54.9% ethanol by volume.

The branched hemicellulose B was examined for homogeneity by electrophoresis using 0.1 M sodium tetraborate-sodium chloride as

electrolyte. The result was not completely satisfactory as there were small tails left behind.

The monosaccharide composition of the branched hemicellulose B was determined quantitatively and qualitatively by gas liquid chromatography. The sample was first hydrolysed in sulphuric acid solution, after which the monosaccharides obtained were reduced to the corresponding alditols by sodium borohydride and subsequently converted into acetyl derivatives. Methyl-α -D-glucopyranoside was added as an internal standard. It was found that each monosaccharide resulted in only one peak, which proved that no epimerisation took place during reduction with sodium borohydride. When varying amounts of alditol acetates were injected into the chromatograph, it was also found that the relationship between the peak areas and the weight of injected acetates were linear. The areas of the peaks were measured and the different components of sugar were calculated. The branched hemicellulose B fraction B contained 16.65% arabinose, 46.94% xylose and 13.43% hexose (see section 3.3 p.61).

The uronic acid content of the polysaccharide was determined quantitatively by the specific colour reactions for sugar and hexuronic acids with carbozole sulphuric acid and also with anthrone sulphuric acid. The absorptions of sugar and uronic acids were measured at 530 and 585 mμ by the spectrophotometer. It was found that branched hemicellulose B fraction B contained 17.8% uronic acid.

Examination of the acid hydrolysate of the polysaccaride on paper chromatographs showed the presence of xylose, arabinose, glucose, a trace of galactose and uronic acids. The neutral components were separated from the acidic components by passing the solution through ion exchange resins. The neutral sugars were further separated by partition chromatography on a cellulose column using butanol half saturated with water as the developing solvent. In this manner, xylose, arabinose, glucose and galactose were separated. The identity of xylose and arabinose was confirmed by converting them into their charateristic crystalline p-nitrophenylhydrazone derivatives. The acidic sugar was eluted from the Dowexl - X_4 by alkali and then converted to free acid. The product on examination on the paper chromatogram, was found to be a single component and had the same R_F as the marker, D-glucuronic acid. Therefore, the acidic component of the branched hemicellulose B fraction B was probably D-glucuronic acid. Its identification was confirmed by preparing the D-glucuronic acid Brucine salt. The values obtained for optical rotation, melting point and mixed melting point were within the ranges of value obtained from the literature.

A portion of branched hemicellulose B fraction B was methylated twice with dimethyl sulphate and sodium hydroxide, after which it was dissolved in dimethyl formamide and methylated twice with methyl iodide and barium hydroxide. After another two treatments with methyl iodide and silver oxide, the methylated acidic

polysaccharide was now completely soluble in chloroform. The final product was a clear, yellow syrup, with an optical rotation in chloroform of $[\alpha]_D^{20} - 105^\circ$. This large negative value indicates a high proportion of β-glycosidic linkages of the main chain.

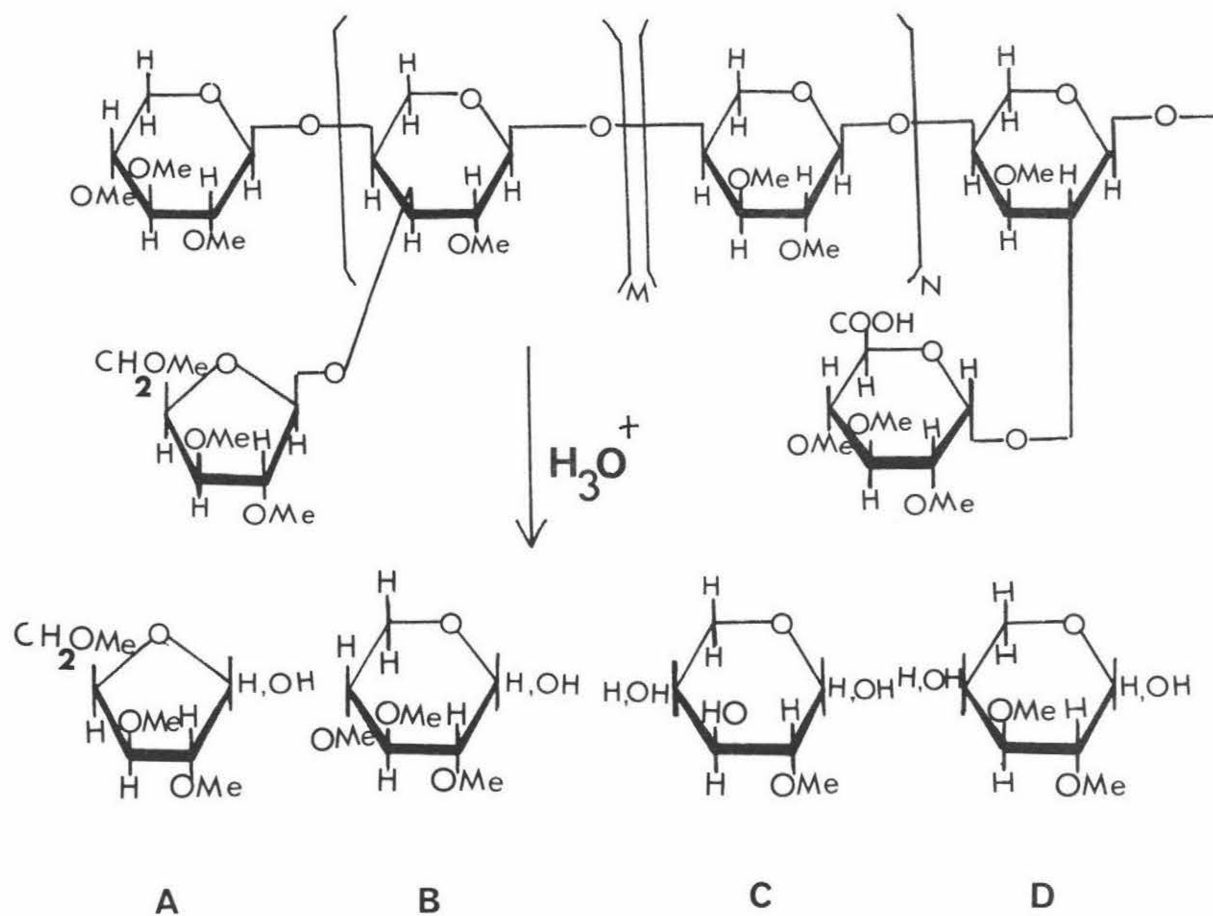
The methylated branched hemicellulose B was subjected to methanolysis, with 3% methanolic hydrogen chloride. After neutralisation, the glycosides were hydrolysed to the corresponding reducing sugars and these were resolved on a cellulose column. The mixture was found to contain 2,3,di-o-methyl-D-xylose, 2-O-methyl-D-xylose, 2,3,4, tri-o-methyl-D-xylose and 2,3,5 tri-o-methyl-L-arabinose. The 2,3,di-O-methyl-D-xylose was characterised by conversion into its crystalline anilide, while the other components were identified by qualitative chromatography, optical rotation and melting point of the crystalline sugars.

A portion of the hydrolysate was successfully examined by gas-liquid chromatography. A typical gas chromatogram is shown in Fig. 8 (see section 3.4,p. 71). Five peaks were observed, four of which were identified. Peak A corresponded to the major component, 2,3, di-O-methyl-D-xylose, peak C was 2-O- methyl-D-xylose. Peak D and E were identified as 2,3,4, tri-O-methyl-D-xylose and 2,3,5-tri-O-methyl-L-arabinose respectively (by comparing the retention times). The areas of the peaks were measured and the molar ratio of the sugars was obtained (see Table 16, P. 86).

FIGURE 2

Methylated Branched Hemicellulose B

And Hydrolysis Products



A. **2,3,5** - tri - **0** - methyl - L - araboturanose.

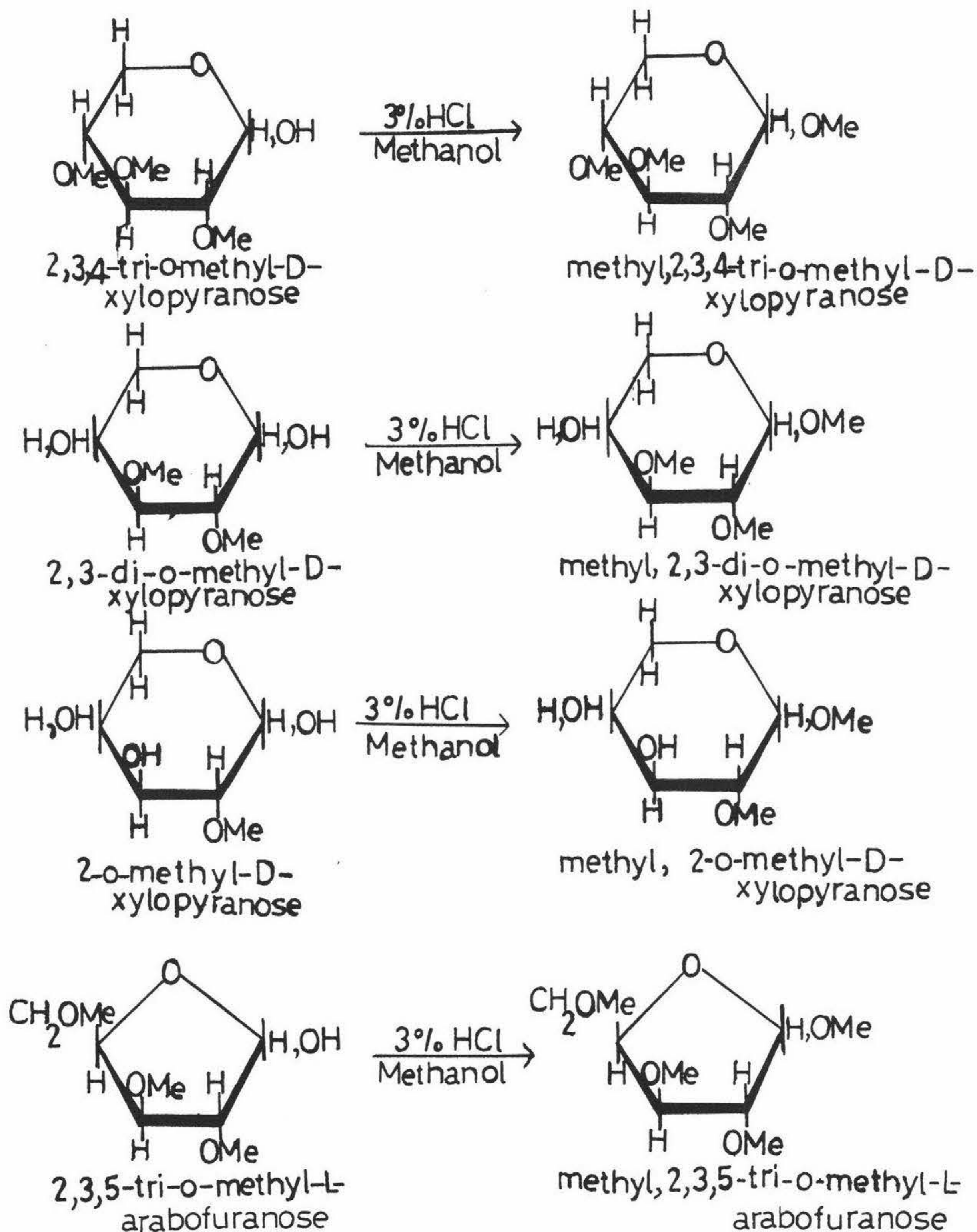
B. **2,3,4** - tri - **0** - methyl - D - xylopyranose.

C. **2-0** - methyl - D - xylopyranose.

D. **2,3** - di - **0** - methyl - D - xylopyranose.

FIGURE 3

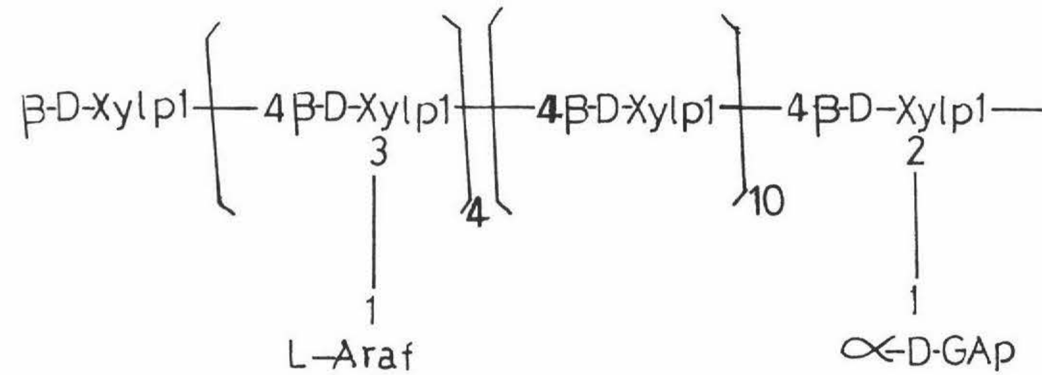
Methanolysis of Methylated Branched Hemicellulose B



The relatively large proportation of 2,3-di-O-methyl-D-xylose produced from the methylated polysaccharide (see Table 16 , p. 86) indicated that the main portion of the hemicellulose was composed of xylopyranose residues linked together through position 1 and 4, while the high negative rotation of the methylated polysaccharide suggested that the anhydroxylose units were present in the β configuration. The 2,3,4 tri-O-methyl-D-xylose evidently originated from the non-reducing end group of the polysaccharide. The presence of 2,3,5 tri-O-methyl-L-arabinose indicated that the hemicellulose, like many otheres, containing L-arabinofuranose units linked glycosidically as non-reducing end units. The ready cleavage of the arabinose unit by mild acid hydrolysis was evidence for the furanose form of the sugar. The 2-O-methyl-D-xylose is derived from units of xylose which form branch points in the molecule, these units are joined through position 3 in addition to position 1 and 4. The uronic acid has been confirmed as D-glucuronic acid, linked to the main chain at position 2 by α glycosidic linkage. There is no trace of 3-O-methyl-D-xylose in the hydrolysate of the methylated polysaccharide, this could be due to the incomplete methylation of the branched hemicellulose B.

On the basis of the molar ratios of the cleavage products of the methylated polysaccharide, it is possible to propose a structure for the branched hemicellulose B consisting of 16 D-xylopyranose units linked 1-4 by β glycosidic bonds, with approximately four 2 mono-O-methyl-D-xylopyranose units for every ten 2,3 di-O-methyl

-D-xylopyranose units. D-glucuronic acid is joined as a single terminal side chain to the xylose unit of the main structure by α 1-2 glycosidic bond. The L-arabinofuranose unit proabaly occurs in the side chain since L-arabinose is the first sugar obtained on hydrolysis. This unit is linked to every fourth D-xylose at position 3.



Xylp = Xylopyranose, Araf = Arabofuranose, GAp = Glucopyranosyluronic acid.

FIGURE 4. Proposed structure for branched Hemicellulose B of Tall Fescue (*Festuca arundinacea*).

CHAPTER III

METHODS AND RESULTS

Melting point determinations were carried out using a Gallenkamp electrical melting point apparatus. Optical rotations were determined using a Hilger standard polarimeter. In the uronic acid determination, the absorption of sugar and uronic acids were measured by SP 500 series 2 ultraviolet and visible spectrophotometer. Microanalyses were carried out using Zeisel's method.[133]

3.1 Extraction of Hemicellulose B from Tall Fescue

The grass was extracted in batches of 200 g.

Extraction Procedure

(A) Preliminary Extraction

The dried grass (200 g) was extracted for eighteen hours with azeotropic ethanol-benzene (4 l, 1:2) in a macro-soxhlet. The extracted material was air dried and weighed (168.6 g).

(B) Water-soluble Polysaccharides Extraction.

The extracted grass (168.6 g) was suspended in water (12 l) at 60°C for thirty minutes with constant stirring and then filtered. The insoluble material was washed with water, dried in an oven at 40°C and weighed.

(C) Pectin Extraction

The water-insoluble material (132.6 g) was extracted by stirring with 2% EDTA (3 l) (sodium form adjusted to pH 6.7 with sodium hydroxide), at 70 °C for two hours. The extraction procedure was repeated three times. The residue was washed several times with distilled water, then dried at 40°C and weighed (121.6 g).

(D) Deproteinization

The extracted material left from pectin procedure (121.6 g) was extracted with 0.5% pepsin in 0.1 N HCl (5 l) for eighteen hours at 46°C. The residue was washed with distilled water until the supernatant was clear then dried and weighed.

(E) Delignification

The residue (85.5g) was suspended in water (2.2 l) and glacial acetic acid (5.7 ml) and chloramine T (30 g) were added slowly to the suspension. The beaker covered with a watch glass was held in a boiling water bath, with occasional stirring for two hours. The suspension was filtered while still warm through a glass filter and washed twice with 95% ethanol. A boiling 3% solution of ethanolamine (500 ml) was left in contact with the residue on the glass filter for two minutes, then suction was applied. The residue was washed again with 95% ethanol, then with water (three times). The whole process was repeated twice

more. The residue was then dried and weighed.

(F) Hemicellulose Extraction

The delignified residue (73.7 g) was extracted with 10% KOH (deionised water) (1.5 l) under nitrogen with stirring for fifteen hours at 25°C. (The nitrogen used was deoxygenated by bubbling through a solution of pyrogallol (15 g) in 50% sodium hydroxide (100 ml). The suspension was centrifuged and the residue was washed with deionised water (1 l) and centrifuged again. The extract and washing were combined.

Hemicellulose A

The extract was adjusted to pH 4.9 with glacial acetic acid and allow to stand overnight. The precipitate which formed was removed by centrifugation, suspended in water, and freeze dried hemicellulose A (5.3 g).

Hemicellulose B

The acidified supernatant was poured into 95% ethanol (4 l) and allowed to stand until a precipitate formed. The precipitate which was collected on a nylon gauze did not completely dissolve in deionised water and so was centrifuged at 10,000 g for fifteen minutes. The clear brown supernatant was poured off and the residue was re-dispersed in deionised water and re-centrifuged at 10,000 g. The supernatants were combined and the residue freeze dried as for hemicellulose B (5.9 g).

The supernatant (500 ml) was adjusted to pH 2.5 with 1 M HCl and poured into 95% ethanol (2 l). The precipitate was collected and redissolved in water. The solution was again adjusted to pH 2.0 with 1 M HCl and poured into 95% ethanol (1 l). The precipitate was washed with 80% ethanol twice and then washed with 95% ethanol until free of chloride ions. The hemicellulose B was dissolved in a minimum volume of water and freeze dried (7.6 g).

(G) Fractionation of Hemicellulose B [87]

The hemicellulose (7.6 g) was dissolved in calcium chloride solution (S.G.1.3) (360 ml) and clarified by a short centrifugation at 20,000 g. a 3% aqueous solution of I_2 (18 ml) and 4% aqueous solution of KI (24 ml) were added to the solution. The dark blue precipitate which formed was left to settle for two hours and then collected by centrifugation at 20,000 g. The clear brown supernatant was neutralised with sodium thiosulphate and poured, with stirring, into five volumes of ethanol to precipitate the branched polymer.

Branched Hemicellulose B

The precipitate was dissolved in 0.1 M MCl (45 ml) and reprecipitated with ethanol (250 ml). The polysaccharide obtained was filtered on a nylon gauze, washed with ethanol and ether, dissolved in a minimum volume of deionised water and freeze-dried (6.3 g).

Linear Hemicellulose B

The dark blue precipitate containing the linear polymer was washed with calcium chloride solution (100 ml) containing 15% iodine-potassium iodide solution. The residue was dissolved in hot water (150 ml), the iodine was neutralised with sodium thiosulphate and the polymer reprecipitated by pouring into five volumes of ethanol. To remove calcium, the precipitate was dissolved in 1 M KOH, neutralised with 1 M HCl and again poured into five volumes of ethanol. The precipitate obtained was dissolved in a minimum amount of water and freeze-dried (0.6 g).

(H) Cellulose Residue

The residue from the hemicellulose extraction was washed with hydrochloric acid until neutralised, then washed with water until the effluent was above pH 5. The residue after centrifuging was suspended in a minimum quantity of water and freeze-dried (53.9 g).

3.2 Assessment of Homogeneity of Branched Hemicellulose B

(A) Fractionation using Fehling Solution

The branched hemicellulose B (7.6 g) was dissolved in 500 ml of distilled water. 20% NaOH solution was added dropwise until all the polysaccharide was dissolved. Freshly prepared Fehling solution was added to the polymer solution until precipitation

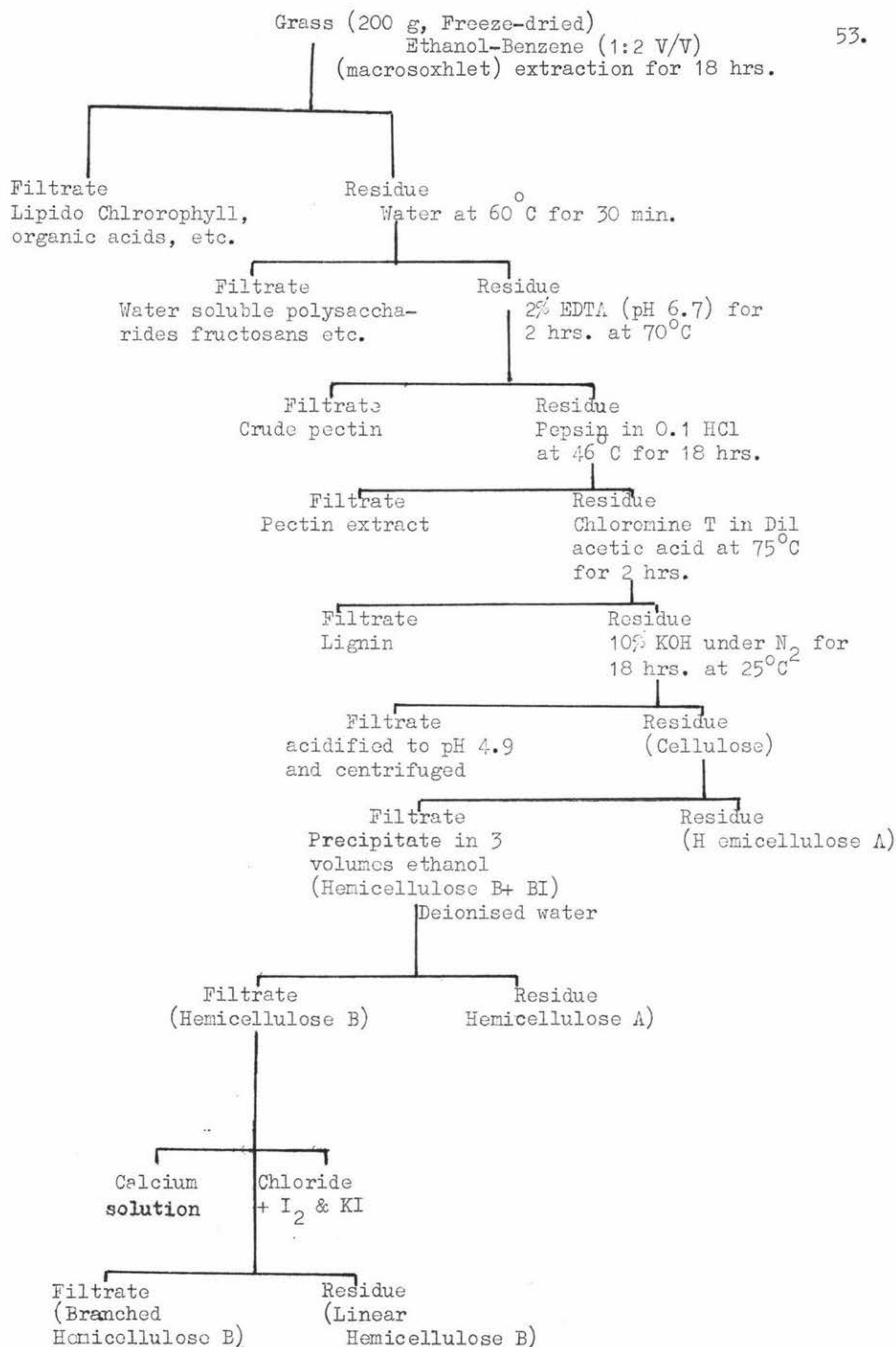


Figure V. Scheme for Extraction of Polysaccharides in Grass.

of the copper complex was just complete. (A large excess of Fehling solution should be avoided since the precipitated "copper complex" is sometimes soluble in excess reagent). The precipitate (Fraction A) was collected by filtration after 4 hours, washed with water, and decomposed by maceration with ethanol at 0° containing 5% (v/v) of concentrated hydrolchloric acid. The residue was washed with ethanol until the washing gave a negative test to chloride. The residue was dissolved in a minimum quantity of deionised water and freeze-dried (2.08 g).

The filtrate from fraction A was neutralized with acetic acid and dialysed against tap water for twenty four hours. The dialysate was concentrated to a small volume under reduced pressure and poured into ethanol (500 ml) at 0°. The precipitate formed was still blue green in colour, which indicated the presence of copper ions. This precipitate was collected by centrifugation, then washed with water and decomposed by maceration. The remaining polysaccharide was washed with ethanol unitl the washing gave a negative test for chloride ions. This white material was dissolved in a minimum amount of water and freeze-dried (3.57 g).

(B) Fractional Precipitation with Ethanol

Fractionation of Hemicellulose B (Fraction B)

The hemi ellulose (2.5g) was dissolved in distilled water to give solution (150 ml) which was adjusted to pH 7 by the dropwise addition sodium hydroxide solution. The insoluble substance was

TABLE VII Composition of Tall Fescue (*Festuca arundinacea*)

(g/200 g moisture-free original grass)

Ethanol-Benzene soluble materials	32.4
Water-soluble polysaccharides	36.0
Pectin	11.0
Crude Protein	36.1
Lignin	11.8
Residue (mainly cellulose)	53.9
Hemicellulose A	5.3
Hemicellulose B I	5.9
Hemicellulose B	7.6

FIGURE 6

Per Cent of Hemicellulose B Precipitated at Various Alcohol Concentration

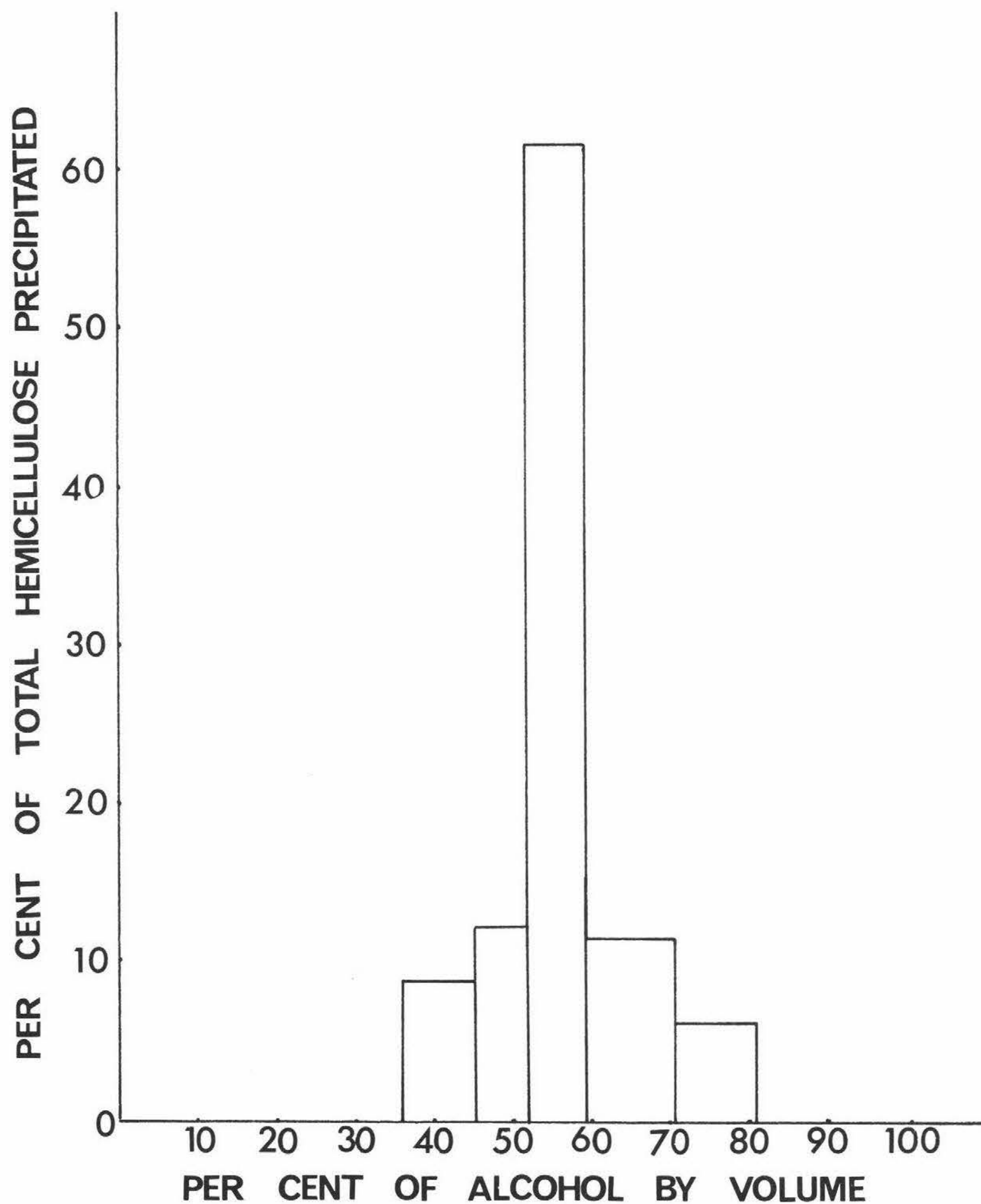
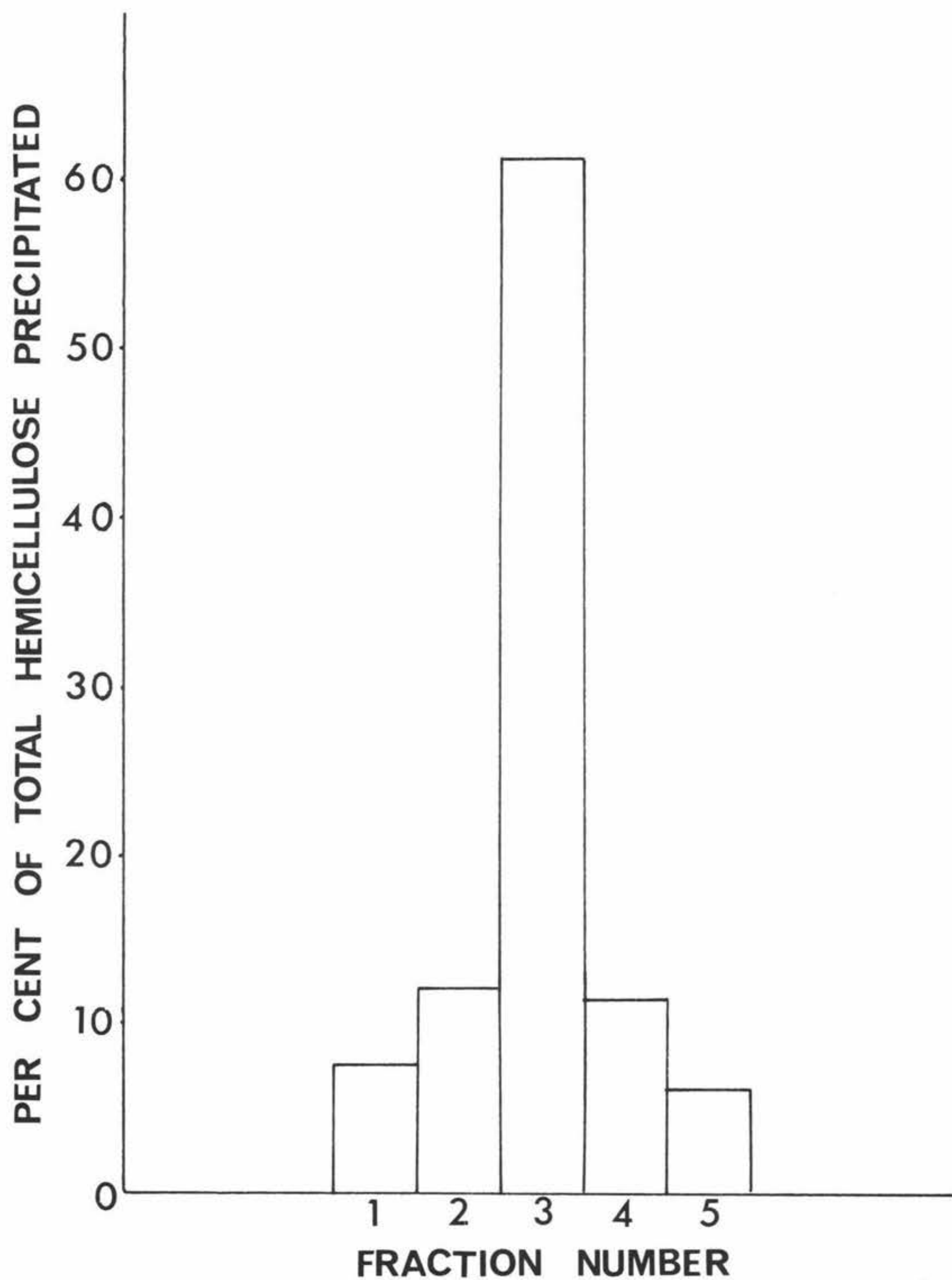


FIGURE 7

Per Cent of Hemicellulose B in Each Fraction



removed by centrifugation at 3000 r.p.m. Ethanol was added dropwise from the burette with stirring to incipient turbidity. After standing for five minutes, the dispersion was centrifuged. The precipitate was washed with ethanol several times then dissolved in distilled water and freeze-dried. Ethanol was added dropwise to the supernatant as before until it became milky white. This insoluble matter was isolated as before and the precipitation procedure was repeated until a concentration of 85.0% ethanol was attained. From table it can be calculated that 86.40% of the hemicellulose was recovered from the starting substance. The precipitation curve was also shown in Fig. 6 & 7 (p. 56, 57).

(C) Zone Electrophoresis

The strip supports (glass wool paper 35 cm x 40 cm) were marked with a pencil at the middle (20 cm) from one end (anode) to indicate the starting line. The strip was then dipped in borate buffer (0.05 M sodium tetraborate decahydrate, pH 9.2) and blotted between sheets of blotting paper using rubber wringer roller which was drawn across the blotter without additional pressure. The polysaccharide dissolved in borate buffer with small amount of 1% NaOH was applied to the strip support by a fine pipet so that 5 - 10μl of the solution containing about 10-50 of the substance was run onto the starting line of the damp support as a thin band not greater than 5 cm in length and as narrow

as possible.

The strip support was suspended horizontally in a tank. The two electrodes compartments each hold approximately 300 ml of buffer (.05 M $Na_2B_4O_7 .10H_2O$; pH 9.2), and the cooling fluid is water.

The electrophoresis was carried out at 1300 V and approx. 100 m A for $1\frac{1}{2}$ hours, the strip support was then removed and dried in an oven at about 100°C for 20 minutes and then sprayed with the spraying reagent consisting of 1 g of p-anisidine hydrochloride, and then heating again at 100°C for half an hour. The polysaccharides were shown up as yellowish zones.

(D) Electrophoresis of dyed polysaccharides on cellulose acetate

Dyeing Procedure

To a solution of polysaccharide (50 mg) in water (5.0 ml) was added a freshly prepared solution of the dye (Procion Red M2B or procion Blue M3G) (50 mg) in water (5 ml). After standing for five minutes sodium chloride was added, either as a solid or as 1 ml of concentrated solution, to give a final concentration of 2%. Thirty minutes later sodium carbonate was added to give a final concentration of 0.1%. The mixtures were set aside for 18 hours (overnight) to allow most of the unreacted dye to be hydrolysed. The reaction mixtures were clarified by

centrifugation or diluted to reduce viscosity and were then added directly to the column (2.5 x 30 cm) of Sephadex G-15 or G-25. Elution of the columns with water produced two widely separated colored zones. The dyed polysaccharides, excluded from the gels, were eluted in the void volume; salts and unreacted dye were retarded. To avoid contamination by inorganic salts, which were eluted more rapidly than the unreacted dye, care was taken to collect only the strongly coloured portion of the polysaccharide eluate. The dyed polysaccharides were then recovered from the eluates by freeze-drying.

Electrophoresis

The Gelman Sepraphore III cellulose acetate strip (2.5 x 15 cm) was soaked in the buffer solution (0.1 M sodium tetraborate - sodium chloride solution) for five minutes. The strip was then pressed between two sheets of Whatman 3 MM filter paper to remove excess buffer and the starting line was marked with a pencil. Samples (1% solutions) were applied as thin lines by using fine glass capillaries. The stip was then positioned in a Shandon Electrophoresis chamber so that the starting line was midway between the terminals which were 10 cm apart. Voltage was applied, 250-250 V, 80 mA, and migration of the bands was followed visibly. The optimum time for all separations was 4 minutes and higher potential gradients (up to 500 V/cm) gave no improvement over the conditions cited above, Poorer results were obtained when the starting line was positioned nearer to either terminal.

When the electrophoresis was finished, the strips were removed and dried immediately in a stream of hot air. If this was not done, the bands diffused and became distorted. Both hemicellulose B fraction A and fraction B showed strong coloured bands with small tails which were left behind.

3.3 Quantitative Determination of Carbohydrates in Hemicellulose by Gas-Liquid Chromatography [107, 135, 136]

Apparatus

The separations were performed on a Varian Aerograph series 1740 (FID) gas chromatograph, equipped with a differential flame ionization detector and automatic servoscribe potentionmetric recorder. The coiled column was constructed from $\frac{1}{8}$" steel and packed with 3% SE - 30.

Nitrogen was used as the carrier gas. The injection port was maintained at 190°C and the detector was matched to that of the recorder by means of a vibrating reed electrometer. The signal from the detector was recorded on a 100 m V full scale strip chart recorder.

Calibration

The pressure was stable and the pressure gauge module was calibrated after the 3% SE - 30 column was installed. The flame ionization detector required carrier gas, hydrogen gas and air for proper operation. The regulators of carrier gas (nitrogen), hydrogen gas and air cylinders were adjusted to be 65 p.s.i., 10 p.s.i. and 20 p.s.i. respectively. The flow rate was adjusted to 20 ml/min. by using soap-bubble flowmeter, and the gas flow was 50 - 60 on the flowmeter.

Procedure

Approximately 20 mg of polysaccharide was hydrolysed for 22 hours with 0.125 M H_2SO_4(10 ml) in a stoppered tube in an oven at 100°C. The hydrolysate was neutralised to pH 6 - 8 with saturated Ba $(OH)_2$ and then 9 - 10 mg of Me - α -D-glucopyranoside was added as a standard. After mixing by stirring for a few minutes, the Ba (SO_4) was centrifuged down and approximate 30 mg of sodium borohydride was added to reduce the aldoses to their corresponding alditols.

After leaving the solution overnight, an excess of cation

exchange resin (Dowex 50 W - X8) was added (0.5 g). The mixture was rotated for about 20 minutes then the supernatant was decanted and filtered. The resin was washed several times with distilled water and these supernatants were also filtered. The combined solution was then evaporated to dryness in a rotary evaporator. Borate ion was removed as volatile methyl borate by three co-distillation with methanol (20 ml).

The residue was acetylated with a mixture of pyridine/acetic anhydride (1:1 V/V, 4 ml) in a boiling water bath for 12 mins. and then evaporated almost to dryness on a rotary evaporator. The syrup was taken up several times in ethyl acetate and re-evaporated to dryness. The remaining syrup was dissolved in ethyl acetate (2 ml) for injection into the gas chromatograph.

Separation

A suitable quantity of the sample in ethyl acetate (0.4 - 0.6 µl) was introduced by means of a Hamilton microlitre syringe into a heated injection tube where they were vaporized. The initial column temperature was 144°C, and the programming was started immediately after injection. After the peak of Me-α-D-

glucopyranoside was through, the temperature was then increased to 170 ^{o}C.

Result

Since no integrator was avaiable, the peaks areas were cut out and measured by weighing on a micro-balance (Mettler H 20). The typical chromatogram of a hemicellulose hydrolysate is shown in Figure 8, p. 71. The retention times and peak area/weight ratios of the alditol acetate was given in Table

3.4 Uronic Acid Determination

(A) Carbozole Method

Solutions:

B is a solution containing the unknown polysaccharide 50 - 100µg/ml uronic acid.

C is a standard solution containing 50µg/ml D-glucuronic acid.

d is a solution containing 200 µ g/ml of sugar (arabinose, xylose and glucose).

1 ml samples of each solutions B, C and d were placed in an

TABLE VIII FRACTIONAL PRECIPITATION OF BRANCHED HEMICELLULOSE B FRACTION B

Fraction number	ml of ethanol added	% of ethanol by volume	Weight of precipitate in g	% of total precipitate
1	100.2	40.0	0.1943	8.79
2	150.8	50.1	0.2611	12.04
3	183.3	54.9	1.3307	61.58
4	280.7	65.2	0.2463	11.57
5	850	85.0	0.1325	6.02

TABLE IX CONVERSION FACTORS FOR THE DETERMINATION OF SUGAR COMPOSITION FROM THE PEAK AREA

Sugar	Conversion Factor
Xylose	0.775
Arabinose	0.741
Hexose	0.813

TABLE X Retention time of fully acetylated glycitols relative to methyl-α-D-glycopyranoside penta acetate

Sample	Relative Retention Time
Arabitol-penta acetate	0.706
Xylitol-penta acetate	0.794
Methyl-α-D-glycopyranoside penta acetate	1 (standard)
sorbitol-hexa acetate	1.471 indistinguishable using column
galacitol-hexa acetate	SE-30 1.471

TABLE XI   Arabinose composition of branched hemicellulose B

Weight of the peak area of Arabinose (in g)	Weight of the peak area of standard (in g)	Ratio = weight of area of Arabinose / weight of area of standard	Weight of standard added (in mg)	Correction factor	Product of ratio, weight of standard and correction factor	Weight of hemicellulose B (in mg)	Arabinose composition %
0.2741	0.4715	0.5813	8.88	0.741	3.82500	22.69	16.86
0.2941	0.5047	0.5827	8.88	"	3.83421	22.69	16.90
0.2063	0.4544	0.4540	9.84	"	3.31031	20.32	16.29
0.2171	0.4497	0.4828	9.56	"	3.42014	20.54	16.65
0.1926	0.3815	0.5048	9.75	"	3.64705	21.70	16.81
0.1889	0.3878	0.4871	9.67	"	3.49030	21.30	16.39

Average 16.65

TABLE XII   Xylose composition of branched hemicellulose B

Weight of the peak area of xylose (in g)	Weight of the peak area of standard (in g)	Ratio = Weight of area of xylose / Weight of area of standard	Weight of standard added (in mg	Correction factor	Product of ratio, weight of standard and correction factor	Weight of Hemicellulose B (in mg)	Xylose composition %
0.7239	0.4715	1.5353	8.88	0.775	10.56593	22.69	46.57
0.7688	0.5047	1.5233	8.88	"	10.48335	22.69	46.20
0.5739	0.4544	1.2630	9.84	"	9.63163	20.32	47.40
0.5845	0.4497	1.2998	9.56	"	9.63022	20.54	46.89
0.5155	0.3815	1.3512	9.75	"	10.21000	21.70	47.05
0.5236	0.3878	1.3502	9.67	"	10.11874	21.30	47.51

Average 46.94

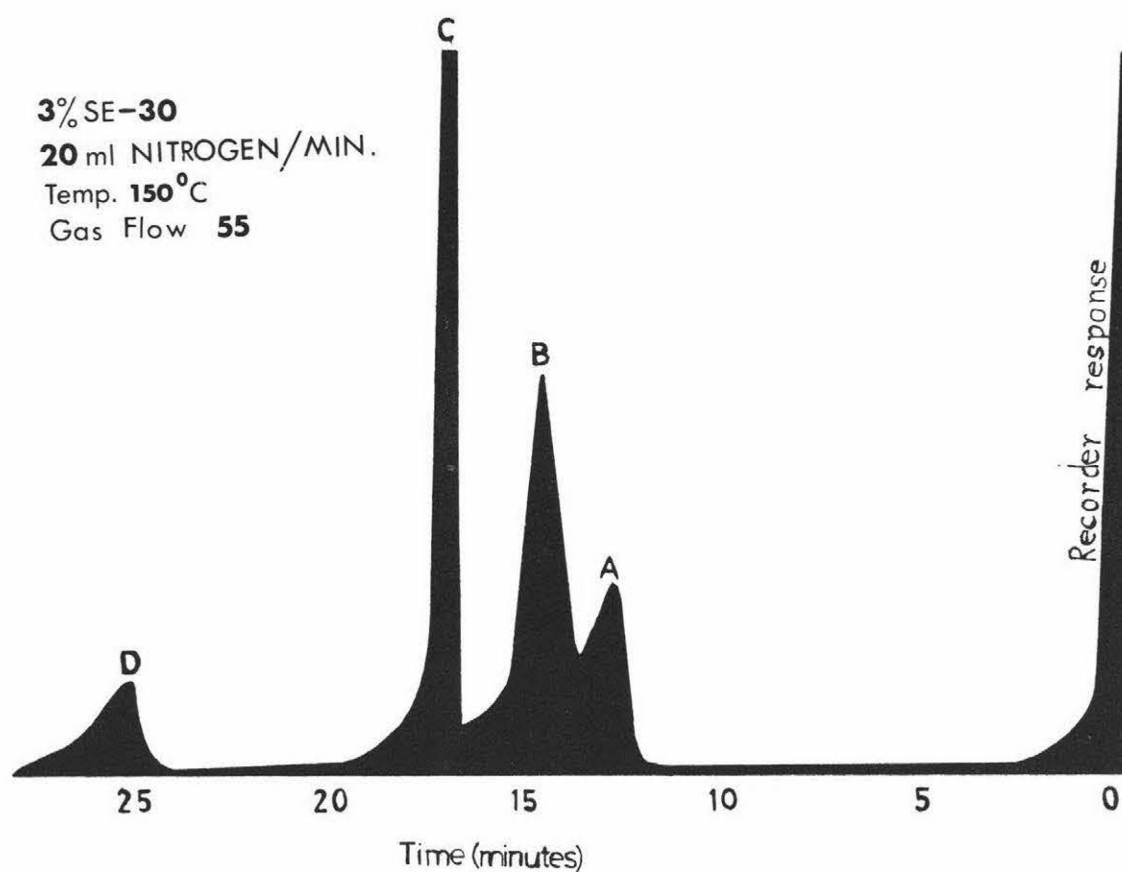
TABLE XIII Hexose composition of branched hemicellulose B

Weight of the peak area of Hexose (in g)	Weight of the peak area of standard (in g)	Ratio = weight of area of Hexose / Weight of area of standard	Weight of standard added (in mg)	Correction factor	Product of ratio, weight of standard and correction factor	Weight of hemicellulose B (in mg)	Hexose composition %
0.2067	0.4715	0.4384	8.88	0.813	3.16500	22.69	13.95
0.2068	0.5047	0.4097	8.88	"	2.95780	22.69	13.04
0.1612	0,4544	0.3548	9.84	"	2.83837	20.32	13.97
0.1507	0.4497	0.3351	9.56	"	2.60449	20.54	12.68
0.1435	0.3815	0.3761	9.75	"	2.98125	21.70	13.74
0.1387	0.3878	0.3577	9.67	"	2.81213	21.30	13.20

Average 13.43

FIGURE 8

The Separation by Gas-Liquid Chromatography of Monosaccharides as Alditol acetates.



A. Arabinose.

B. Xylose.

C. Methyl - α - D- glycopyranoside.

D. Hexose.

ice bath, and concentrated sulphuric acid (6 ml) was added slowly to each down the side of the tubes. The solutions were agitated with a glass rod and heated for exactly twenty minutes in a boiling water bath. The mixtures were then cooled rapidly and a 0.1% alcoholic solution (0.2 ml) of carbazole was added with shaking. After a few minutes, a pink-purple colour appeared and the intensity of this increased for two hours and then remained stable. The tubes were left in the dark at room temperature for 3 hours. The absorption was then measured at 530 m μ by spectrophotometer.

(B) Anthrone Method

Solutions:

a is a solution containing 100 μ g/ml of sugar (arabinose, xylose and glucose).

b is a solution containing 200 μ g/ml D-glucuronic acid.

A is a solution containing the unknown polysaccharides, 50 - 100 μ g/ml uronic acid.

2 ml samples of each solution a, b, and A were pleaced in an ice bath, after fifteen minutes of cooling, a 0.2% solution of

anthrone in concentrated sulphuric acid (4 ml) was added slowly to each down the side of the tubes. The mixtures were stirred with a glass rod, and the tubes were sealed with a glass stopper and heated in water bath at 92°C for eight minutes. The solutions were cooled and the tube were left in the dark at room temperature for thirty minutes. The absorption was then measured at 585 m by UV spectrophotometer. The quantity of uronic acid was determined in mg/ml by the following formula:

$$\frac{Ba - Ad}{\frac{Ca}{50} - \frac{bd}{200}} : 1000$$

B = 0.55		a = 1.23
c = 0.268		b = 1.78
d = 0.27		A = 1.13

Total uronic acid was 89µ g/ml

Polysaccharide was 1 µ g in 2 ml (from above),

i.e. 500 µ g/ml

$\therefore$ % uronic acid in the polysaccharide $= \frac{89}{500} \times 100 = 17.8\%$.

Paper Chromatography

The separation of sugars using paper chromatography was carried out on Whatman No. 1 and 3 MM filter papers with the

following solvent systems: (A) ethyl acetate-glacial acetic acid - formic acid-water (V/V, 18:3:1:4), (B) n-butanol-ethanol-water (V/V, 4:1:5), (C) an azeotropic mixture of 2-butanone and water, b.p. 74-75°C. The developed chromatogram were sprayed with a 3% solution of p-anisidine hydrochloride in ethanol. R_x values of neutral and acidic oligosaccharides refer to rates of movement relative to xylose. R_G and R_x values of methylated sugars refer to rates of movement relative to 2,3,4,6 tetra-O-methyl-D-glucose and 2,3,4,6 tetra-O-methyl-D-galactose respectively.

3.5 Separation and Identification of Monosaccharides from the Product of Hydrolysis of Branched Hemicellulose B (Fraction B) [137]

The hemicellulose (1 g) was dissolved in 0.125 M sulphuric acid (170 ml) and the mixture was heated in a stoppered flask at 100°C for twenty two hours. The solution was cooled and brought to pH 6 by the addition of saturated barium hydroxide and barium carbonate. The barium sulphate formed was removed by filtration and the filtrate was concentrated to about 80 ml on a rotary evaporator and then passed through Amberlite IR-120 (hydrogen form) ion exchange resin. Uronic acids were absorbed from the eluate by passing it through a column of Dowex 1-X_4 (OH^-) exchange resin, after which the resin was washed with water (1.5 l) until a negative anthrone test showed that all sugars had been removed. All the washings were combined together and concentrated in a rotary evaporator at 40°C to a syrup which was the neutral

sugars (A) (0.7g).

The uronic acids were then eluted from Dowex 1 - X_4 (OH^-) exchange resin by 1 M sodium hydroxide and were converted to the free acid form by removal of sodium ions on Amberlite IR-120 (H^+) exchange resin, the filtrate was evaporated under reduced pressure at 40°C to a syrup (B) (0.12 g).

Separation of Sugars in (A)

The neutral sugar fraction was analysed by paper chromatography using solvent A and was found to contain a mixture of xylose, arabinose and glucose. These were further separated by chromatography on a cellulose column using butanol half saturated with water as the solvent.

3.5.1 Quantitative Analysis of Mixtures of Sugars by Partition Chromatography on Column of Powdered Cellulose[138, 139, 140, 141, 142].

The mixture of meutral sugars (A) was added as a concentrated aqueous solution onto a column of cellulose (3.5 x 40 cm) and eluted with butanol half saturated with water. The eluate was fractionated into fractions of about 15 ml. After the separation had been completed, the distribution of the component sugars in the tubes was determined by paper chromatography using solvent A and the p-anisidine hydrochloride spray. Four components were apparent which corresponded to xylose, arabinose, glucose and galactose. The eluates from tubes containing the same components were combined

to give five fractions. The first group (100.3 mg) contained pure xylose, while the second group (48.1 mg) contained a mixture of xylose and arabinose. Group III (33.5 mg) was pure arabinose, group IV (28.1 mg) was pure glucose. Group V (9.0 mg) seemed to be a mixture of glucose and galactose.

Group II was chromatographically separated on Whatman No. 1 3 MM filter paper using solvent A. Approximate zones, indicated by marker strips on both sides of the paper, were cut and the components extracted with water. The more rapid-moving component Ia (R_x 1.00) and the slow moving components Ib (R_x 0.85) were identified as xylose and arabinose respectively.

Identification of xylose

The syrup (100.3 mg) was recrystallized several times from ethanol to give the white crystal, m.p. 145°C, lit., m.p. 145-148° $[\alpha]_D^{20}$ + 15.6 (C.5 in water) lit., $[\alpha]_D$ + 93.6 — + 18.8 (C.4 in water).

p-Nitrophenylydrazine (180 mg) dissolved in alcohol (1 ml), was added to a solution of xylose (90 mg) in water (08 ml). The mixture was warmed on a water bath and water was added until a turbidity appeared. The crystalline nitrophenylhydrazone separated on standing and was filtered off and washed with water. Recrystallization upon aqueous alcohol gave the characteristic xylose-p-nitrophenylhydrazone, m.p. and mixed m.p. 153°C, lit., m.p. 156°C

TABLE XIV Results of the separation of sugars by partition chromatography

Fraction number	tube number	Yield mg	R_x	Components identified
1	35-47	100.3	1.00	Xylose
2	48-60	48.1	1.00 0.85	Xylose Arabinose
3	61-75	33.5	0.85	Arabinose
4	93-118	28.1	0.56	Glucose
5	119-159	9.0	0.56 0.53	Glucose Galactose

Solvent A : ethyl acetate-glacial-acetic acid-formic acid-water (18:3:1:4).

R_x : The distance between the centre of the sugar spot and the starting line with relative to that of standard xylose.

Identification of arabinose

This component (33.5 mg), R_x 0.85 was recrystallized several times from ethanol. The white crystalline solid had m.p. 156 - 157°C, lit., m.p. 160°C $[\alpha]_D^{20}$ + 103.1 (C.2 in water), lit. $[\alpha]_D$ + 190.6 — + 104.5 (C.4.3 in water).

Arabinose was also confirmed by preparation of p-nitro-phenylhydrazone derivate using the same method as that for xylose. Arabinose-p-nitro-phenylhydrazone had m.p. and mixed m.p. 185°C. lit., m.p. 187 - 188°C

Separation of Acids in (B)

Paper chromatography in solvent A showed that the uronic acid had the same R_x (1.10) as the marker D-glucuronic acid. The syrup was cystallised from methanol to give crystals m.p. 163°C lit., D-glucuronic acid m.p. 165°, $[\alpha]_D^{20}$ + 19.4, lit. $[\alpha]_D$ + 11.7 — + 63.6 (in water) which were further identified as D-glucuronic acid. Brucine salt derivative, m.p. 154 - 155°, lit., m.p. 156-157°C $[\alpha]_D^{20}$ - 13.3 (C 1.5 in water), lit., $[\alpha]_D^{20}$ - 15.1 (in water).

3.6 Methylation of Branched Hemicellulose B by Methods after Haworth, Kuhn and Purdie [143,144,145].

Haworth Procedure [124,146,147,148,149].

40% sodium hydroxide (80 ml) and dimethyl sulphate (32 ml) were added to a constantly stirred solution of pure branched hemicellulose B (2 g) dissolved in water (25 ml), at room

temperature over a period of six hours in such a way that the solution was always alkaline, stirring was continued for another eighteen hours. The mixture was methylated again as above but the temperature was raised to 50°C. The solution was transferred to a beaker which was heated on a sand bath. A precipitate which was the partly methylated polysaccharide formed on the surface. It was removed and dissolved in boiling water (50 ml), the solution was dialysed against tap water for twenty-four hours, and evaporated at 40°C in vacuo to a syrup (1.6 g).

Kuhn Procedure [127, 150]

The partially methylated polysaccharide (1.6 g) was dissolved in dimethyl formamide (50 ml) and the temperature was raised to 30°C. Methyl iodide (22 ml) and barium hydroxide (22 g) were added during three hours. After another twelve hours of stirring the partially methylated polysaccharide had precipitated. Chloroform (50 ml) was added and stirring was continued for another twelve hours. The supernatant was decanted from the barium hydroxide and the latter was extracted twice with chloroform (50 ml). The chloroform extracts were combined with the above supernatant which was then washed with an equal volume of water (6 times). The chloroform solution was centrifuged (3000 r.p.m.) and the water layer removed with a pipette. The extract was dried over anhydrous sodium sulphate, filtered and evaporated in vacuo at 30°C to a syrup (1.5 g).

The syrup was methylated again, by dissolving it in dimethyl formamide (50 ml) and adding methyl iodide (20 ml), silver oxide (15 g) over a period of twenty four hours to the constantly stirred solution at 45°C. After another twelve hours of stirring, the reaction mixture was centrifuged (4000 r.p.m.) and the supernatant was decanted from the precipitate, the later was washed with dimethyl formamide (25 ml) and chloroform (25 ml), and these two fractions were combined with the above supernatant. Chloroform (175 ml) was added and the solution was shaken with potassium cyanide (5 g) to remove silver ions. The filtered solution was washed with equal volumes of distilled water (8 times) and dried over anhydrous sodium sulphate, then evaporated in vacuo to a syrup (1.4 g) (Found : OCH_3, 26.07% calculated for $C_7H_{12}O_4$ (2 OCH_3 grs.) OCH_3, 38.7%).

Purdie Procedure[151,152,153.]

The partially methylated branched hemicellulose B from the above was dissolved in methyl iodide (25 ml) and the solution was refluxed with silver oxide (2.5 g) which was added in six portions during twelve hours. The mixture was refluxed for another twelve hours. The excess methyl iodide was distilled off under reduced pressure and the residue was extracted three times with acetone (40 ml). The filtered solution was concentrated to give a light brown liquid which was further methylated by the Purdie Procedure. The final syrup was dissolved in chloroform

(150 ml) and shaken with potassium cyanide (5 g) to remove silver ions. The solution was filtered and washed with equal volumes of distilled water (6 times) before being concentrated to a syrup (1.2 g) $[\alpha]_D^{20}$ - 105 (C. 1 in chloroform). (Found OCH_3) 32.76% calc. OCH_3 38.7%.

3.6.1. Hydrolysis and Separation of Methylated Branched Hemicellulose B[23,154,155,156,157].

The methylated branched hemicellulose B (1.0 g) was dissolved in 3% methanolic hydrochloric acid (100 ml) and refluxed for six hours. After neutralisation with silver carbonate, centrifugation and filtration, the solution was reduced in vacuo at 30 °C to a syrup (0.8 g). A portion of the syrup (0.4 g) was dissolved in 0.5 sulphuric acid (40 ml) and heated in a sealed flask at 100 °C for eight hours. The hydrolysate was neutralised with barium carbonate and barium hydroxide. After filtration, the solution and washings were passed through ion exchange resins, IR - 45 (OH) and IR - 120 (H) respectively, and then evaporated in vacuo at 30 °C to a syrup (0.26 g). Paper chromatography in solvent Band C showed that there were four major components, which corresponded to 2,3, di-methyl-D-xylose, 2 mono-methyl-D-xylose, 2,3,4, tri-methyl-D-xylose and 2,3,5 - tri-methyl L-arabinose. The syrup (0.25 g) was separated on a cellulose column (42 x 3.5 cm), irrigated with 2-butanone-water azeotrope and the effluent collected in 15 ml. aliquotes. Paper chromatography with solvent B and C

permitted the grouping together of aliquots with the same composition.

On the basis of R_X and R_G values, and colour reaction. Fraction I (52.1 mg) appeared to be 2,3,5 - tri-methyl-L-arabinose. Fraction II (22.9 mg) seemed to be 2,3,4, tri-methyl-D-xylose. Fraction III (73.1 mg) contained, 2,3, di-methyl-D-xylose and Fraction IV (88.0 mg) seemed to be a mixture of 2,3, di-methyl-D-xylose and 2-methy-D-xylose. Fraction IV was chromatographically separated on sheets of Whatman 3 MM filter paper with irnigant C. Marker strips at the sides of the paper indicated the zones which were cut out and the components extracted. The most rapid-moving component (R_G 0.65) was 2,3, di-methyl-D-xylose and the slow-moving component (R_G 0.23) was 2-mono-methyl-D-xylose.

Fraction I

Chromatography of the syrup (52.1 mg) showed the presence of 2,3,5 - tri-methyl-L-arabinose, which had $[\alpha]_D^{20}$ - 36.5 (C. 1.0 in water), lit., $[\alpha]_D^{25}$ - 36 (C.0.5 in water)[157] and had R_X values of 1.30 (solvent C) 0.96 (solvent B) and R_G values of 1.02 (solvent C) and 0.94 (solvent B). Further evidence of its identification was obtained by gas chromatography.

Fraction 2

This syrup (22.9 mg) $[\alpha]_D^{20}$ + 17.8 (C. 1.2 in water) Lit., $[\alpha]_D^{25}$ + 18.1 (C. 1.0 in water)[157] Chromatography showed the presence of 2, 3, 4, tri - methyl - D- xylose

TABLE XV Hydrolysis products from methylated branched hemicellulose B

Fraction number	Tube number	Weight (mg)	Solvent C R_X	Solvent C R_G	Solvent B R_X	Solvent B R_G	Component
1	5-18	52.1	1.30	1.02	0.96	0.94	2,3,5 tri-methyl L-arabinose
2	19-21	22.9	1.24	1.00	0.94	0.92	2,3,4 tri-methyl D-xylose
3	22-38	73.1	0.80	0.65	0.82	0.79	2,3 dimethyl D-xylose
4	40-80	88.0	0.28	0.23	0.51	0.47	2-mono-methyl D-xylose

Solvents: (C) 2-butanone-water azeotrope

(B) n-butanol-ethanol-water (4:1:5)

R_X is relative to 2,3,4,6 tetra-O-methyl D galactose

R_G is relative to 2,3,4,6 tetra-O-methyl D-glucose

(R_G 1.00) (solvent C), (R_G 0.92) (solvent B). Attempt to prepare crystalline derivative failed.

Fraction 3 Identification of 2,3, di-methyl-D-xylose

This component (73.1 mg) had $[\alpha]_D^{20}$ + 21.5 (c.0.5 in water), lit., $[\ ]_D^{25}$ + 25 (C. 1.0 in water)[156] + 24[157], + 23[154]. Paper chromatography showed R_F values of 0.80 (solvent C) and 0.82 (solvent B), and R_G lit., o.64, 0.68[155], 0.61[154] (solvent C) and 0.79[155] (solvent B).

This fraction was refluxed for two hours in a solution of 5 ml absolute methanol containing 1 ml of aniline. Upon evaporation of solvent, remaining aniline was removed by azeotropic distillation with water. After the last trace of aniline was removed, a crystalline derivative appeared which, upon recrystallisation from ethyl acetate methanol, had m.p. 110°C, lit., m.p. 124 C[157]:

Fraction 4 Identification of 2-mono-methyl-d-xylose

This fraction (88.0 mg) had $[\alpha]_D^{20}$ + 20.2 (C. 1.0 in water) lit., $[\alpha]_D^{25}$ + 21[155], + 21.4[154]. Paper chromatography showed R_F values of 0.47 (solvent B) 0.23 (solvent C), lit., 0.47[155] (solvent B), 0.23[157], 0.22[156].

A portion of this fraction was dissolved in absolute methanol and concentrated to a syrup which gave crystals on recrystallisation from ethyl acetate methanol, m.p. 126°C lit., m.p. 131°C[156].

3.6.2 Quantitative and Qualitative Determination of Methyl Ether Monomers by Gas Liquid Chromatography

A portion of the methylated branched hemicellulose B (1.0G) was dissolved in 3% methanolic hydrochloric acid and refluxed for six hours (see p. 81). After neutralisation with silver carbonate, the solution was concentrated to syrup in vacuo at 30°C. A small portion (20 mg) of the syrup was dissolved in chloroform and the solution was injected into the gas chromatography (the same column as used before, see p. 61).

The peak were identified by injection of known samples into the chromatography under the same operating conditions as for the product from hydrolysis of methylated hemicellulose B, and the retention times of the peaks were compared.

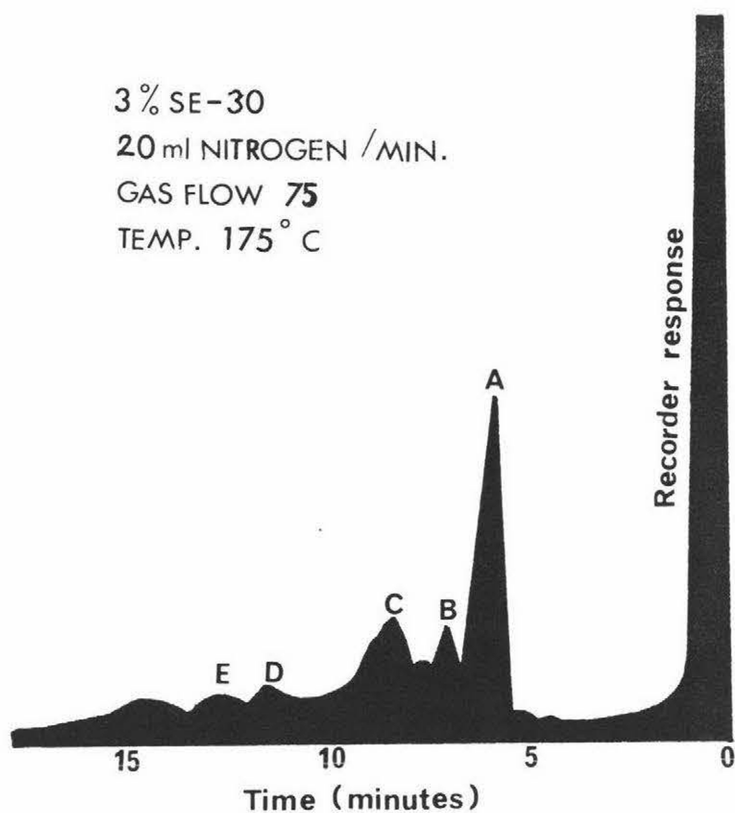
The molar ratio of the sugars were obtained by measuring the individual peaks then worked out the relative ratio.

TABLE XVI Products from hydrolysis of methylated branched hemicellulose B.

Sugar	Molar ratio
2,3, di-O-methyl-D-xylose	9.5
2-O-methyl-D-xylose	4.4
2,3,4, tri-O-methyl-D-xylose	1.1
2,3,5, tri-O-methyl-L-arabinose	1.1

FIGURE 9

Gas-Liquid Chromatography of Methylated Methyl Glycosides



A. 2,3, di-o-methyl-D-xylopyranose.

B. unidentified.

C. 2, mono-o-methyl-D-xylopyranose.

D. 2,3,4, tri-o-methyl-D-xylopyranose.

E. 2,3,5, tri-o-methyl-D-xylopyranose.

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