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

1971

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.

ACKNOWLEDGEMENTS

The author would like to thank Professor E.L. Richards for his supervision and for creating the opportunity for carrying out this research.

Professor G.N. Malcolm, Dr G.G. Midwinter and Dr L.F. Molloy are gratefully acknowledged for their help and encouragement.

Thanks are also due to my fellow research students Rex Gallagher, Alan Danks and Wayne Sharman for much help and to the other staff of the Chemistry and Biochemistry Department for their assistance in many ways. To Miss Heather Knapp for typing this thesis.

Finally, the author wished to extend her grateful to the Government of New Zealand for a Colombo Plan Scholarship.

CONTENTS

CHAPTER I

INTRODUCTION

CHAPTER II

DISCUSSION

CHAPTER III

METHODS AND RESULTS

LIST OF FIGURES

LIST OF TABLES

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	
Ligin containing the non-carbohydrate material of the cell wall	Hemicellulose Shorter chains containing: Xylose Units Mannose Units Uronic acid units Methoxyl groups Acetyl groups and (possibly galactose and arabinose units)	Cellulose 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 hemicallulose 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 galactomannans 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	41
Brasenia schreberi Gmel (watershield)	22	47	9	L-Rhamnose 25 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

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	Refer-ences
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.....	61
			3	
			:	
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		

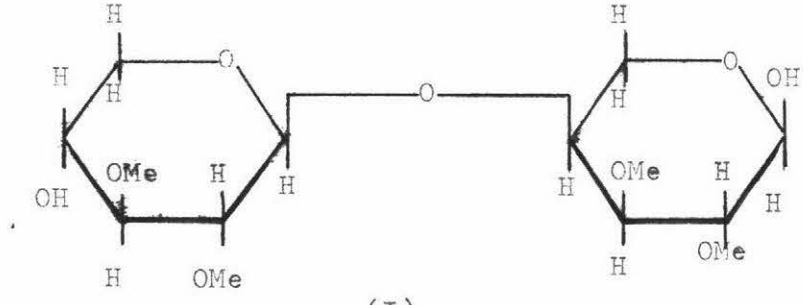
[a] D-Xylp = D-xylopyranose, L-Araf = L-arabinofuranose, D-Gp = D-glucopyranose and Galp = galacto-pyranose residues

[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

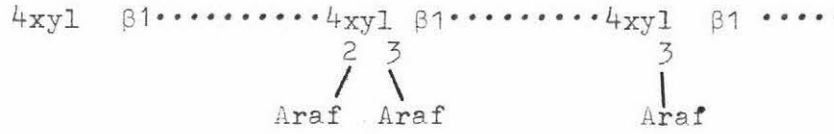
β-D-xylp-(1-4)-β-D-xylp)$_x$-(1-4)-(β-D-xylp)-(1-4)-(β-D-xylp)y-
3
|
1
β-D-xylp-(1-4)-β-D-xylp)$_2$-(1-4)-β-D-xylp

xylp = xylopyranose x+y+z= 75 ± 5

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]

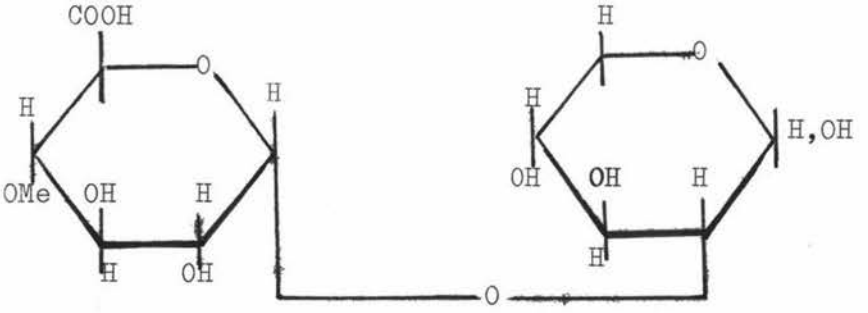
Bishop and Whitaker[20] have isolated an enzyme from myrothecium verrucaria which hydrolyses linear chains of β-(1-4) linked D-xylose units. Application of the enzyme to wheat straw hemicellulose yields among other products, L-arabinofuranosyl - (1-3) β-D-xylo-pyranosyl - (1-4) D-xylose.

β L Araf (1-3) β D Xyl p (1-4) β D Xylp

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.



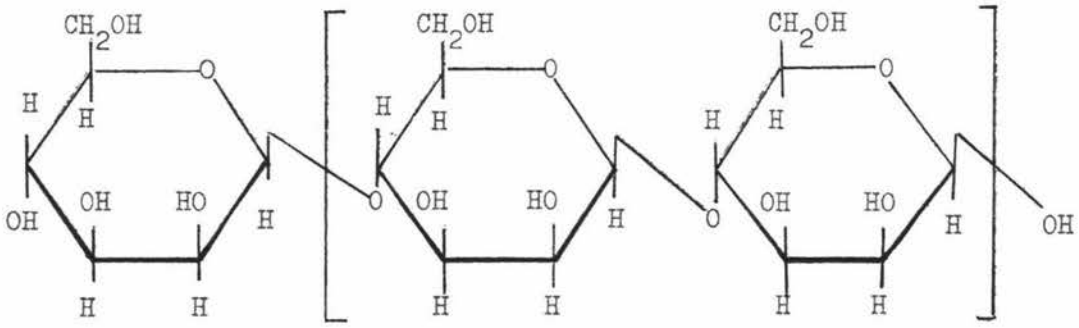
D-glucuronic acid or its 4-methyl ether are most commonly linked to position 2 of D-xylose, although similar aldobiouronic acids containing (1-3) wheat straw) and (1-4) linkages (corn cob) have also been observed.

In the structural study of hemicellulose of Lucerne, Aspinall and McGrath[23] have found that the polysaccharide contains an essentiallylinear 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) Arabino-galactans

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 mechanical 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. Delignification 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 carbohydrates. 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) Hemicellulose Extraction

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 Hemogeneity 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

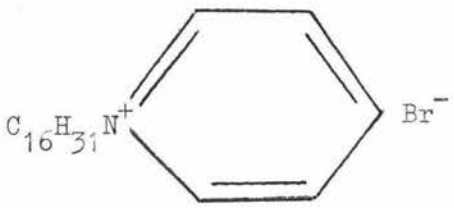
While electrophoresis and ultracentrifugation are excellent methods for fractionating polysaccharide they are usually only applicable to the separation of microquantities.

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.

$C_{16}H_{31}N^{+}(CH_3)_3\ Br^{-}$ (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

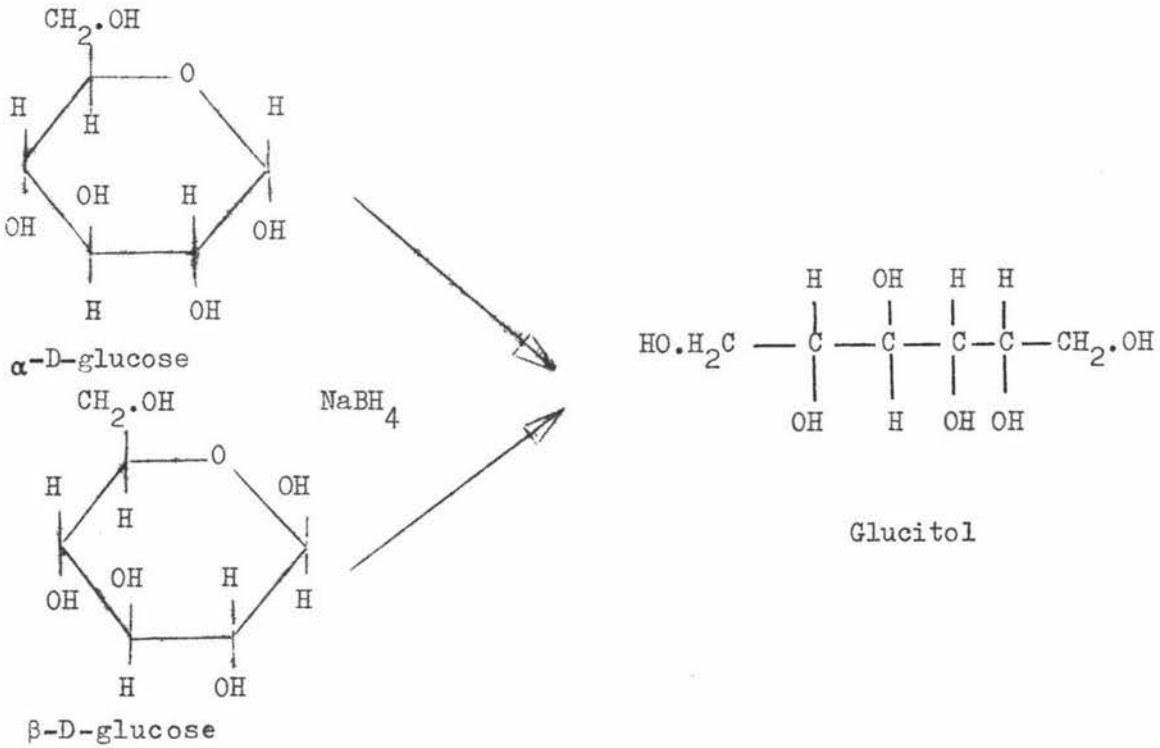
arsenite and basic lead acetate are the most effective electrolytes for separating reducing sugars, basic lead acetate is the most effective for separating sugar alcohols, while borax is the best for simple glycols. Dudman and Bishop[101] also found that electrophoresis of dyed polysaccharides can be 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.

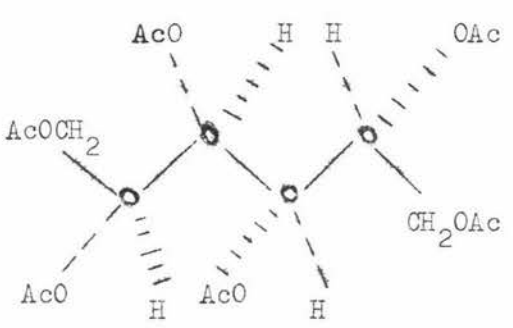
The sugars can be converted into their derivatives quantitatively, but problems arise when separating monosaccharides by gas liquid chromatography. Anomerisation and ring isomerisation can result in the formation of as many as four glycosides from each monosaccharide. Each of these glycosides will produce 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.



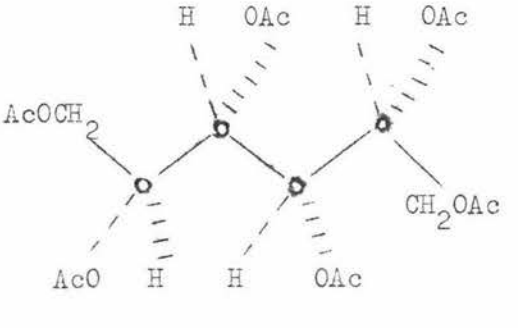
Initially trimethylsilyl derivatives were formed by the method of Sweeley, Bentley, Makita and Wells[107] but, as in their investigation, it was found that arabitol/xylitol and galactitol/glucitol mixtures could not be separated on an SE-52 column (Silicone gum rubber, phenyl). Consequently, alditol 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.



(V)



(VI)

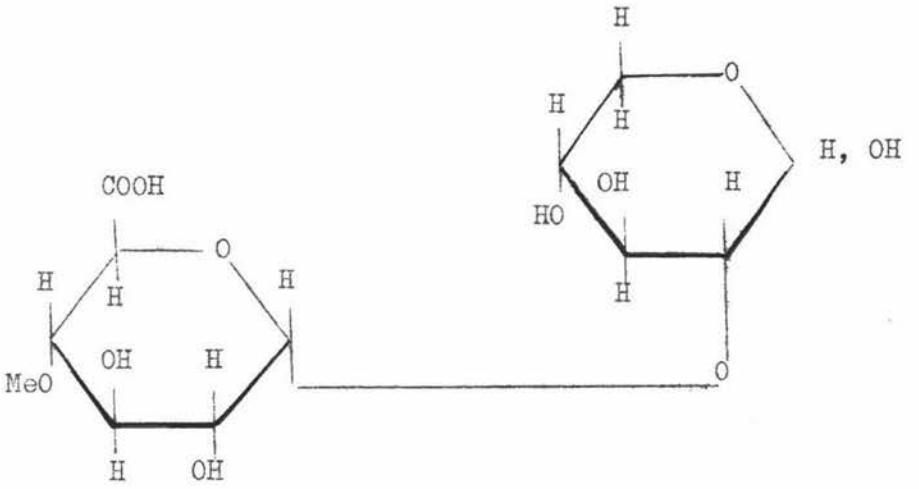
On the basis of these 'rules' of Gunner et al, it is reasonable to expect the affinity of each acetylated molecule for the liquid phase to be roughly similar, hence their difficulty of resolution.

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