A STUDY OF PECTIC MATERIAL FROM THE BARK OF AMABILIS FIR (ABIES AMABILIS)

by

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GENERAL INTRODUCTION

The existence of a water-insoluble precursor of pectin has been known since 1848 when Fremy reported the occurrence of a "pectose" (1). This material was insoluble in water and even in cold concentrated hydrochloric acid but was transformed by heating in dilute acid solutions into a water-soluble pectin similar in properties to the pectin which had been discovered in plant juices. Pectin has now been isolated from many sources, although its chemistry is not yet completely known. In addition to the well-known use of pectin as food and for various industrial purposes, pectin is now rapidly finding more use in medicine (2). It can be employed directly as a therapeutic agent, as a supplement or potentiator of a drug. Pectin has the ability to inhibit the growth of certain viruses. It is injected with hormones, sulfa drugs, and antibiotics, and is also used in diarrhea therapy.

It is known that bark contains a considerable amount (5-10%) of pectin while wood contains only 0.5-1%. Because of the growing uses of pectin and also in view of the importance of a better bark utilization, a more detailed knowledge of the structure of bark pectin is of interest.

The present study is concerned with the isolation and determination of the constitution of pectin from the bark of amabilis fir (Abies amabilis (Dougl.) Forb.).

The common pulpwoods are grouped into two general classes, namely gymnosperms and woody angiosperms. The commercially important gymnosperms are all conifers and are often referred to as "softwoods". Similarly, the arborescent angiosperms are termed "hardwoods". Amabilis fir, a softwood, is the most abundant of the firs in the Pacific northwest and

forms extensive pure forests in many localities. It attains its largest proportions in the Olympic Mountains in western Washington, where mature trees are generally from 140 to 160 ft. in height and 2 to 4 ft. in diameter. In mixed stands this species is commonly associated with Sitka spruce, Douglas fir, grand fir, western hemlock and western red cedar.

HISTORICAL INTRODUCTION

Bark - The term "bark" is used loosely in the nontechnical sense referring to the outer part of the stems and branches encircling the wood. Anatomically, it includes all the tissues outside the vascular cambium to the outermost layers of woody stem or branch. These tissues are classified into primary and secondary tissues, based upon the origin of the cells. The primary tissues are differentiated from apical meristems. In ordinary barks of seed plants, this group of tissues consists of epidermis, cortex and primary phloem. Secondary tissues are differentiated from two special meristems, namely the vascular cambium and the cork cambium. The secondary phloem is derived from the former. This tissue is sometimes called "bast", or "inner bark". In general, bark on a young branch or stem is composed of both primary and secondary tissues.

Barks from the common pulpwoods were long considered as a waste. Presently, considerable efforts are being made to find new uses for bark. Some barks have been tested for making low grade pulp or modified board. Attention is being paid also to the by-products and the problems of complete bark utilization.

The over-all chemical composition of many barks has already been established (3,4). The chemical constituents of bark can be classified into four main groups, namely lignin, cork, polysaccharides and extraneous materials.

Bark polysaccharides can be divided into three groups, namely a) pectic substances which are generally removed by neutral or acid extraction of the bark holocellulose, b) the hemicelluloses which are removed from the remainder by extraction with alkali, and c) cellulose

which is the residue left after all extractions. Gums and mucilages present in some barks are also polysaccharides. Bark from autumn-felled trees also contains starch in appreciable amount.

Bark Hemicelluloses - Much of the early investigations on bark hemicelluloses were concerned with the general information as to the nature of the constituent sugar residues. Schwalbe and Neumann (5) in 1930 first recognized the presence of a well defined hemicellulose fraction in bark, when they detected considerable amounts of readily hydrolyzable hexosans and pentosans in the inner bark of spruce, pine and red beech. Buston and Hopf (6) in 1938 reported that ash bark contained approximately 20% hemicelluloses which on hydrolysis gave mannose, galactose, arabinose and galacturonic acid. In 1947 Cram and his co-workers (7), in a study of the chemical composition of western red cedar bark observed that the largest carbohydrate component of the outer bark was glucose, corresponding to 37.3% of the bark. In 1955 Chang and Mitchell (8) reported the chemical composition of many pulpwood barks. All of them contained residues of glucose, galactose, mannose, arabinose and xylose, with glucose as the major component.

A systematic investigation of the structural features of bark polysaccharides was made by Painter and Purves (9) in 1960. They isolated six chemically distinct groups of polysaccharides from the inner bark of white spruce (Picea glauca). On hydrolysis these yielded, respectively, (a) galactose, glucose, and mannose, (b) xylose and arabinose, (c) galactose, (d) arabinose, (e) glucose, and (f) galacturonic acid. The fraction containing galactose, glucose and mannose in the ratio 1:2:9 had $[\alpha]_D$ -12°. Methylation results suggested that it was a linear chain of β-(1-->4)-linked glucose and mannose residues. Timell (10) pointed

out that the galactose residues might be an integral part of a galactoglucomannan, and not originate from a galactan as assumed by Painter and Purves. The fraction consisting of xylose and arabinose residues, consisted mainly of a (1 --> 4)-linked xylan. Examination of the methylated galactan suggested that it was highly branched, while the identification of 2,3,6-tri-O-methyl-D-galactose suggested the presence of a small amount of β-(1 --> 4)-linked galactan of the type usually associated with pectic materials.

Jabbar Mian and Timell (11) isolated several polysaccharides in a good yield from the bark of white birch (Betula papyrifera). Extraction of the holocellulose with aqueous potassium hydroxide gave a pure 4-O-methylglucuronoxylan in a yield of 27%. This hemicellulose had $[\alpha]_D$ -68° and consisted of a linear backbone of 230 (1 --> 4)-linked xylopyranose residues with approximately one (1 --> 2)-linked 4-O-methyl-α-D-glucuronic acid side chain per ten xylose residues. The material remaining after removal of the xylan, on extraction with sodium hydroxide in the presence of boric acid gave 3.8% of a polysaccharide mixture. The remaining material was pure cellulose (12).

A detailed investigation of the polysaccharides occurring in the bark of several gymnosperm species was started by Timell (10). Four species, namely Amabilis fir (Abies amabilis), Engelmann spruce (Picea engelmannii), Lodge-pole pine (Pinus contorta) and Ginkgo biloba were selected for this study. The hemicelluloses present in the bark of amabilis fir were studied in detail. Extractive-free bark was delignified with acid chlorite, and the resulting holocellulose was successively extracted with hot water, 0.5% aqueous ammonium oxalate, 24% potassium hydroxide and finally with 17.5% sodium hydroxide containing 4% boric

acid, an extraction sequence which had previously been used for various softwoods (13). From the hot water and the potassium hydroxide extracts two apparently similar galactoglucomannans (A and B) were isolated by precipitation with barium hydroxide. The material remaining in solution, after removal of galactoglucomannan B, on further treatments with barium hydroxide gave a pure arabino-methylglucurono-xylan. The sodium hydroxide-borate extract similarly gave an alkali-soluble glucomannan containing minor amounts of galactose residues.

The xylan (14) was shown to consist of at least 124 (1 --> 4)-linked xylose residues, every sixth of which carried a single terminal (1 --> 2)-linked 4-O-methyl-α-D-glucuronic acid unit, while every tenth residue contained (1 -->3)-linked L-arabinofuranose group, attached as a single unit side chain. The two water-soluble galactoglucomannans (15) consisted of a slightly branched framework of at least 80 β-(1 --> 4)-linked D-mannose and D-glucose residues, every tenth of which on the average carried a (1 --> 6)-linked D-galactopyranose unit. The alkali-soluble glucomannan had a linear structure of 70 randomly distributed β-(1 --> 4)-linked glucose and mannose residues, with a few galactose residues existing as single unit side chain and thus forming an integral part of the molecule (16).

Recently three hemicelluloses have been isolated (17) from the bark of Engelmann spruce (Picea engelmannii) based predominantly on residues of xylose, galactose, glucose and mannose. The xylan, obtained in a yield of 4.5% of the extractive-free bark, contained residues of galactose, glucose, 4-O-methylglucuronic acid, arabinose and xylose in a ratio of 6:12:7:10:65. Results obtained on methylation and partial hydrolysis indicated the presence of a β-(1 --> 4)-linked xylose framework

to which 4-O-methyl-α-D-glucuronic acid units were directly attached by (1 --> 2)-linkage. The arabinose residues were present as terminal non-reducing end groups. The glucose-based polysaccharide ("glucan"), obtained in a yield of 1.8%, contained uronic acid, galactose, glucose, mannose and xylose in a ratio of 2:11:57:2:28 and consisted of (1 --> 4)-linked glucose, (1 --> 6)-linked galactose, and (1 --> 4)-linked xylose residues. The glucomannan was obtained in a yield of 2.0% and contained a backbone of (1 --> 4)-linked β-D-glucopyranose and β-D-mannopyranose residues, some of which carried as a side chain an α-D-galactopyranose unit, directly attached to the 6-positions.

Bark Pectins - In addition to hemicelluloses, many barks appear to be very rich in pectic substances. Ash bark (6) contained 7% of pectic material, and balsam bark was found by Hay and Lewis (18) to contain 14% of a "water-soluble mucilage" in addition to other carbohydrates. Sharkov and co-workers published a series of papers (19,20,21) on pectic materials in the inner barks of pine, fir and birch. Pine bast was reported to contain up to 35% of pectin. Anderson and co-workers have isolated the pectin components of both inner bark (22) and the adjacent cambial zone (22,23). Pectic material in a yield of 10% was isolated from the phloem of black spruce (24). Painter and Purves (9) obtained pectin from the inner bark of white spruce in a yield of 7% of the bark. Timell and Jabbar Mian (25) isolated a pectic material (3-4%) from the inner bark of white birch, containing D-galacturonic acid, D-galactose and L-arabinose in a ratio of 66:7:27 and also traces of glucose, xylose and rhamnose units. This was the first attempt at a structural study of a bark pectin. The product could not be resolved by various fractionation methods, but free boundary electrophoresis suggested that it was a mixture of three

different polysaccharides. A methylation study of this heterogeneous product indicated that the galacturonic acid residues were linked together by α-(1 --> 4)-linkages and that L-arabinose existed as non-reducing end group.

Thornber and Northcote (26) made a quantitative determination of the changes that occurred in the composition of a cambial cell during its differentiation and subsequent maturation in four species of trees namely Acer pseudoplantanus, Betula platyphylla, Fraxinus elatior and Pinus ponderosa. The amount of pectic substances per cell had been found to be constant during the formation of the sapwood, but in the heartwood there was an almost complete absence of this fraction (Table 1).

The carbohydrate composition of the pectic substances of the cell walls in the phloem, cambial, sapwood and heartwood regions of these four trees was also determined by the same authors (27). The results are presented in Table 2. It was suggested that the presence of glucans and xylans in pectic substances could be due to the inclusion in those preparations of small amounts of whole woody tissue. The occurrence of rhamnose in pectic substances and also in hemicelluloses was reported. Because of the relatively high percentage of rhamnose it was suggested that it must be a constituent of pectic substances. The cambial region of each tree contained a higher percentage of rhamnose than the phloem, which in its turn had a higher percentage than the xylem.

Present Knowledge of the Chemistry of Pectin - It is known that the isolated pectin is not the true representative of native pectin due to unavoidable changes taking place during extraction. With the knowledge of the structure of isolated pectin and also the possible changes

TABLE 1

Pectic substances in different regions of trees (26)

Percentages of samples after extraction with ethanol-benzene (1:2, v/v)

Species	Phloem		Cambium	Sapwood	Heartwood
	outer	inner			
Ash (Fraxinus elatior)	18.0		6.6	1.4	0.5
Birch (Betula platyphylla)	4.4		18.0	2.6	0.3
Sycamore (Acer pseudoplatanus)	3.1	6.6	15.0	3.8	1.3
Pine (Pinus ponderosa)	10.0		8.5	1.3	1.0

TABLE 2

Carbohydrate composition of the pectic substances prepared from each tree of the four species (27)

All values as per cent

	Anhydro sugar	Phloem	Cambium	Sapwood	Heartwood
Ash	Galactan	6.0	24.0	17.0	6.0
	Glucan	5.5	7.5	19.0	28.0
	Araban	15.0	18.0	9.0	4.0
	Xylan	3.5	2.0	4.5	15.0
	Rhamnan	0.1	0.5	trace	trace
	Uronic anhydride	70.0	48.0	48.5	47.0
Birch	Galactan	7.0	38.5	7.0	6.0
	Glucan	21.0	9.0	26.0	39.0
	Araban	14.1	14.0	8.0	7.0
	Xylan	2.0	3.0	8.0	10.5
	Rhamnan	1.0	1.2	trace	trace
	Uronic anhydride	55.0	34.0	51.0	37.0

(cont'd.)

TABLE 2 (cont'd.)

Anhydrosugar		Phloem		Cambium	Sapwood	Heartwood
		outer	inner			
Sycamore	Galactan	8.5	4.0	35.5	5.0	4.8
	Glucan	28.0	5.0	8.1	15.3	15.7
	Araban	10.0	10.0	12.9	8.1	7.6
	Xylan	8.0	4.2	1.0	6.9	7.7
	Rhamnan	trace	6.0	3.0	1.0	0.5
	Uronic anhydride	45.0	70.0	39.2	64.0	64.0
Pine	Galactan	7.0		23.0	7.0	6.2
	Glucan	11.1		13.5	13.2	12.9
	Araban	18.5		22.0	10.1	14.1
	Xylan	0.5		1.0	1.9	3.9
	Rhamnan	trace		0.5	trace	trace
	Uronic anhydride	62.0		38.0	68.0	62.0

during isolation one can, however, visualize the nature of the native pectin.

Although pectic substances are essentially polymers of D-galacturonic acid, neutral sugars like D-galactose, L-arabinose, and L-rhamnose and sometimes other sugars also are associated with it (48). Earlier studies indicated the existence of such association but did not give any definite proof for it. Even when purified from copper-soluble polysaccharides by precipitation from aqueous solution with cupric ion, the anhydrogalacturonic acid content ranges from less than 80% for carrot to 92% for orange as shown in Table 3 (28). Arabinose, galactose, xylose, and rhamnose have been demonstrated among the hydrolysis products of pectic substances from coffee (29). In sisal pectic acid, Aspinall and Cañas-Rodriguez (30) found the above sugars plus D-glucose, 2-O-methyl-L-fucose, and 2-O-methyl-D-xylose. The pectin isolated from passion fruit peel, however, was found to yield only L-arabinose and L-sorbose in addition to D-galacturonic acid (31). Aspinall and Fanshawe (32) extracted mixtures of pectic substances from lucerne, including a polysaccharide of pectic acid type which was isolated from an ammonium oxalate extract in a pure state. Partial hydrolysis of this pectic acid yielded L-rhamnose, L-arabinose, D-galactose, D-galacturonic acid, traces of fucose, 2-O-methyl-L-fucose, 2-O-methyl-D-xylose, and a mixture of acidic oligosaccharides including 2-O-(D-galactopyranosyluronic acid)-L-rhamnose and oligomers of D-galacturonic acid. Anderson and King (33) extracted from the fresh-water green alga Nitella translucens a non-esterified pectic acid which contained 74% of uronic anhydride and on hydrolysis yielded galacturonic acid with galactose, arabinose, xylose and rhamnose in the ratio of 4:6:3:1.

Pectic acids free from neutral sugars are not generally obtain-

TABLE 3

Sugars liberated by hydrolysis of purified pectinic acids

	Anhydrouronic acid (%)	Arabinose	Galactose	Rhamnose	Xylose
Orange	92.1	+	+	+	0
Grapefruit	91.7	+	+	+	0
Lemon	90.4	+	+	+	0
Apple	88.0	+	+	+	+
Fig	87.1	+	+	+	0
Peach	86.8	+	+	+	+
Pea pod	84.8	+	+	+	+
Apricot	83.1	+	+	+	+
Pear	82.6	+	+	+	+
Sugar beet	82.3	+	+	+	+
Avocado	79.0	+	+	+	+
Carrot	76.7	+	+	+	0

able by mild extraction. From some plant materials, however, highly pure pectinic acid almost free from neutral sugars has been obtained (34,35). Bishop (36) extracted with oxalate a pure galacturonan from sunflower heads.

Pectic substances are usually considered as mixtures of three closely associated polysaccharides and it is not yet definitely known whether or not they are linked by covalent bonds. In the light of the earlier studies these three polysaccharides are identified as pectic acid, containing chains of (1 --> 4)-linked α-D-galacturonic acid residues, present partly as a methyl ester, a linear galactan containing chains of (1 --> 4)-linked β-D-galactopyranose residues, and a highly branched araban, containing (1 --> 5)- and (1 --> 3)-linked α-L-arabinofuranose residues (37). Recent investigations, however, definitely indicate that each of these three polysaccharides is not necessarily composed of only one type of sugar. Andrews and co-workers (38) isolated by extracting with hot lime water an araban from sugar beet chips which contained L-arabinose, D-galactose, L-rhamnose and D-galacturonic acid in the ratio of 74:10:3.5 and 5% respectively and smaller quantities of 2-O-methyl-D-xylose, 2-O-methyl-L-fucose, mannose and fucose. A β-(1 --> 4)-linked galactan with 13% galacturonic acid residues has been isolated from spruce compression wood (39). As mentioned before, in many cases it has also been impossible to isolate a pectic acid devoid of neutral sugars.

Aspinall (32) in his detailed structural investigation of the apparently homogeneous lucerne pectic acid discussed the significance of the results obtained. It was pointed out that the main chain of the polysaccharide must be composed of (1 --> 4)-linked α-D-galacturonic acid residues, that L-rhamnose residues might be interposed between blocks of

(1 --> 4)-linked α-D-galacturonic acid residues as in plant gums of the Khaya genus (47), that the exact location of arabinose residues was not clear but that it must be an integral sugar component, and that the D-galactose residues were mainly present as non-reducing end group, some of them probably being involved in (1 --> 6)-linkages.

The methods used in structural hemicellulose chemistry (40) are also applied to structural elucidation of pectin. The most widely used methods are complete methylation of the polysaccharide and subsequent quantitative estimation of the components obtained on hydrolysis, partial fragmentation of the polysaccharide followed by identification of the resulting oligosaccharides, and periodate oxidation. The latter technique is not generally used, however, because of the difficulties caused by the easy over-oxidation (41) of the polyuronides.

Biosynthesis - Bearing in mind the general structural features of pectic acid, galactan and araban, it is hard to visualize the transformation of one of these into another in nature, although the reason for finding them together might be due to some closely related biogenetic route. There are at least three ways by which pentose units might originate in plants (42), namely through C-6 decarboxylation, through C-1 decarboxylation or thrpugh equilibrium reactions in the "ester pool" involving transketolase and transaldolase.

Hirst (43) pointed out in 1942 that the "decarboxylation theory" was still tenable if oxidation and decarboxylation reactions occurred at the monosaccharide level. The simple uronic acid and pentose derivatives thus formed could theoretically be condensed with each other in any order or arrangement possible to give a wide variety of polysaccharides. The fact that such a series of reactions would have to occur in a gel or

water-depleted system creates at least some doubt about this possibility (46).

An investigation on the formation of pectin in berries (44,45) made by Seegmiller and co-workers gave results in agreement with the C-6 decarboxylation theory. Considering the available information in connection with the biosynthesis of pectin and hemicelluloses it has been suggested (42) that both the "ester-pool", and the "glycoside-pool" are involved through a hypothetical bridge in the biosynthesis of polysaccharides. The "ester-pool" consists of ω-phosphate esters of monosaccharides and the "glycoside-pool" may be composed of sucrose, glucose-1-phosphate, uridine diphosphate glycosides and possibly other nucleotides and pentose-1-phosphates.

Present Investigation - As mentioned before, the structures of several hemicelluloses from the bark of amabilis fir have already been established, including an arabino-4-O-methylglucuronoxylan, a water-soluble galactoglucomannan and an alkali-soluble galactoglucomannan. In view of the fact that the present structural knowledge of the pectic acid still leaves some controversial points, the object of the present investigation was to obtain pure pectin from the bark of amabilis fir, resolve it into the constituent polysaccharides and then to determine the structure of each.

RESULTS AND DISCUSSION

The results accompanied by discussion have been presented in three parts. Part I deals with the extraction, purification, fractionation and other preliminary studies on pectin from the fir bark. Part II is concerned with a polygalacturonic acid. The results obtained with a water-soluble pectic acid and also, in some cases, an unfractionated ammonium pectate used for the structural information of the former are discussed in Part III.

PART I

Extraction of Crude Ammonium Pectate

Extraction is an important step in structural studies of plant polysaccharides. During extraction, the chemical composition and structure may be changed by fractionation, degradation and chemical modification. Pectic acid had been reported (49) to give various oxidation products by the action of some delignifying agents. It is possible that most of the reducing groups in the polysaccharide are oxidized to aldonic acid end groups. Zienius and Purves (50) showed that some D-galacturonic acid could be recovered unchanged after treatment with sodium chlorite buffered near pH 2.8 even under conditions that would have oxidized glucose to gluconic acid. It is, therefore, possible that some of the reducing end groups of a pectic polysaccharide can remain after delignification with sodium chlorite. As it is known that the glycosidic linkage of the polygalacturonide is quite resistant to acid hydrolysis, most of these bonds probably remain intact during the preparation of the holocellulose (pH 3-4). On the other hand, the possibility of hydrolysis

of at least some of the acid-labile arabinofuranosidic bonds and of acetate groups cannot be completely excluded. Since it is not possible to isolate and fractionate bark polysaccharides without prior delignification (10), the extractive-free bark of amabilis fir was treated with sodium chlorite and acetic acid to give a holocellulose in a yield of approximately 50%.

The holocellulose was extracted with water at 75-80°C for twelve hours to remove water-soluble polysaccharides, after which the residue was treated with 0.5% aqueous ammonium oxalate for twelve hours at 75-80°C. After concentration of the ammonium oxalate extract and precipitation with ethanol, the crude ammonium pectate ($[\alpha]_D$ +120° in water) was obtained in a yield of 13%. According to Anderson and coworkers (52), decarboxylation can occur when pectic substances are extracted with water and with 0.5% aqueous ammonium oxalate to the extent of 1-3% and 3-8%, respectively. D-galacturonic acid on decarboxylation presumably would give rise to L-arabinose. The steps involved in the isolation of the crude ammonium pectate in the present case were therefore subjected to a closer examination. A small-scale experiment was carried out, using different extraction periods with both water and ammonium oxalate solutions, as seen in Fig. 1. A total of 16 samples of crude ammonium pectates were obtained; the yield and specific rotations are given in Table 4. All of these samples were purified by precipitating the insoluble calcium pectates and then regenerating the ammonium pectates. The purified samples were all found to have almost the same composition, as can be seen from Table 5. The lower specific rotations of these preparations indicated that the latter were probably contaminated with neutral polysaccharides. No attempt was made to purify these samples

FIGURE 1

Procedure for extraction of holocellulose with water and ammonium oxalate solution for different lengths of time

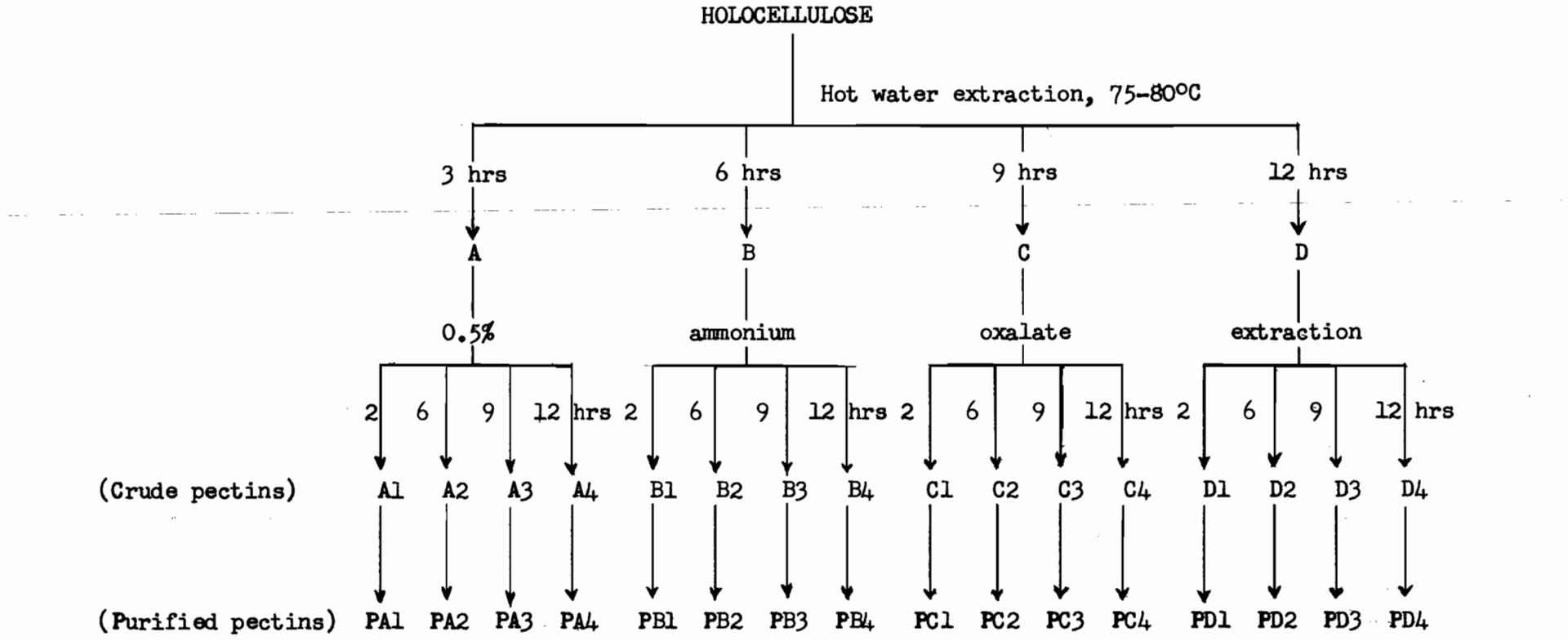
HOLOCELLULOSE
Hot water extraction, 75-80°C
3 hrs
6 hrs
9 hrs
12 hrs
A
B
C
D
0.5%
ammonium
oxalate
extraction
2 6 9 12 hrs
(Crude pectins)
A1 A2 A3 A4
B1 B2 B3 B4
C1 C2 C3 C4
D1 D2 D3 D4
(Purified pectins)
PA1 PA2 PA3 PA4
PB1 PB2 PB3 PB4
PC1 PC2 PC3 PC4
PD1 PD2 PD3 PD4

TABLE 4

Crude ammonium pectates obtained by using different extraction periods

Sample	Yield, % of holocellulose	$[\alpha]_D$ in water, degrees
A1	10.0	+109
A2	10.0	+110
A3	13.0	+ 99
A4	17.0	+ 98
B1	10.0	+ 99
B2	10.0	+105
B3	10.0	+105
B4	10.0	+108
C1	10.0	+105
C2	15.0	+107
C3	10.0	+110
C4	12.5	+110
D1	10.0	+112
D2	13.0	+110
D3	14.0	+109
D4	14.0	+110

TABLE 5

Purified ammonium pectates from the different crude samples

Sample	Yield, % of holocellulose	$[\alpha]_D$ in water, degrees	GalA:Gal:Ara	GalA / Ara
PA1	2.12	+192.2	78.0:5.2:16.8	4.81
PA2	2.28	+191.1	78.0:5.3:16.7	4.67
PA3	2.94	+197.0	76.2:6.1:17.7	4.30
PA4	2.35	+189.1	78.0:6.0:16.0	4.87
PB1	2.18	+187.5	79.5:5.0:15.5	5.12
PB2	2.70	+185.0	79.0:5.0:16.0	4.93
PB3	2.52	+198.1	78.0:5.0:17.0	4.58
PB4	2.75	+189.5	77.0:5.0:18.0	4.27
PC1	2.03	+193.0	76.0:6.0:18.0	4.22
PC2	3.47	+195.0	77.0:6.0:17.0	4.52
PC3	2.32	+198.7	78.0:5.5:16.5	4.72
PC4	3.27	+198.0	78.0:5.0:17.0	4.58
PD1	2.50	+199.2	78.0:5.0:17.0	4.58
PD2	3.15	+196.0	77.0:6.0:17.0	4.52
PD3	3.51	+196.3	76.5:6.2:17.3	4.42
PD4	3.81	+198.9	76.3:5.9:17.8	4.28

further. Considering that the purification steps in all cases were identical and that no marked fall in the ratio of galacturonic acid to arabinose was observed, it was clear that few, if any, of the galacturonic acid groups in the pectin were converted into arabinose during the large-scale extraction. It is possible that little decarboxylation occurred in this case, because not all pectic material was exposed to the extracting agent all the time. Alternatively, decarboxylation might not necessarily give rise to arabinose.

The material extracted with hot water from the holocellulose in a yield of 3.5% gave on hydrolysis galacturonic acid, galactose, glucose, mannose, arabinose and xylose in a ratio of 10:8:20:44:10:8. Free boundary electrophoresis of the crude product in 0.1N sodium chloride (Fig. 2) suggested that it consisted of three components (The main peak was due to the salt boundary). The constitution of a galacto-glucomannan isolated from the water-extract was reported earlier (15). The very low yield (1-2%) of pectin from the water-extract did not permit any detailed study. It was observed, however, that the ammonium pectate obtained from the water-extract behaved on treatment with cation exchange resin as the ammonium pectate isolated by extraction with ammonium oxalate. Evidently, a small portion of the pectin exists in the bark in a water-soluble form instead of insoluble calcium pectate.

Resolution of the Crude Ammonium Pectate

The crude ammonium pectate gave, on hydrolysis, galacturonic acid, galactose, glucose, mannose, arabinose, and xylose in a ratio of 38:8:10:22:12:10 and also minor quantities of rhamnose. The free boundary electrophoresis of this material (Fig. 3) indicated that it was hetero-

FIGURE 2

Free boundary electrophoresis of the water-extract in sodium chloride solution

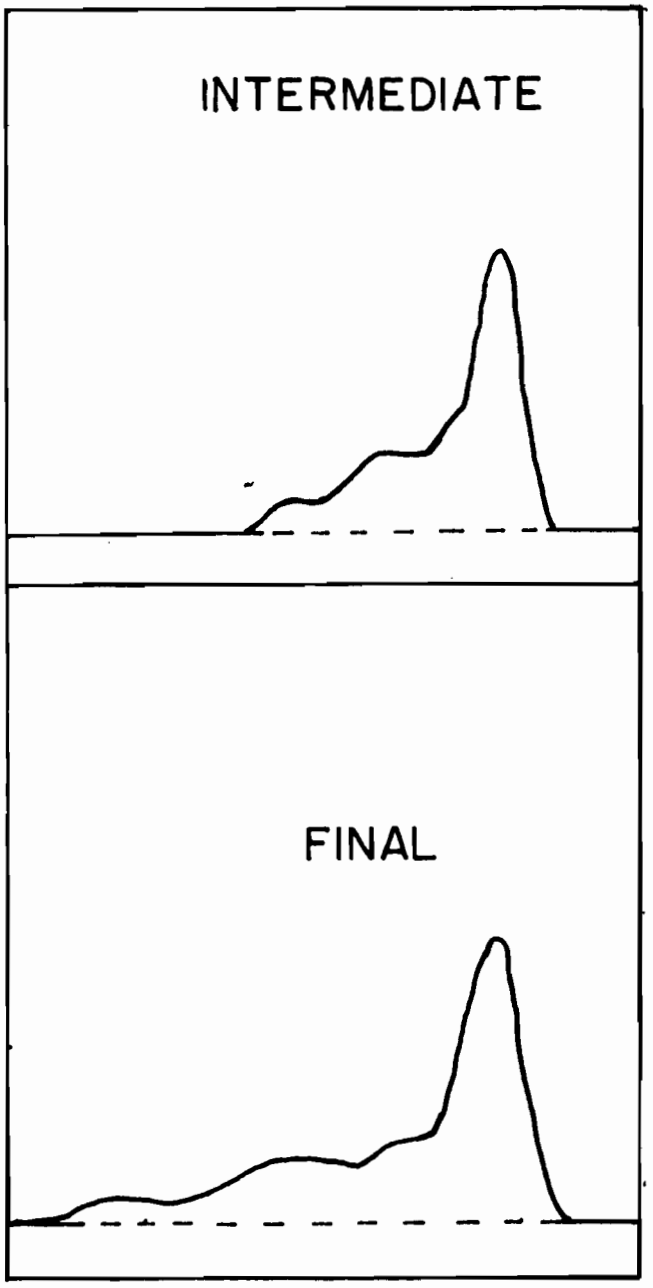
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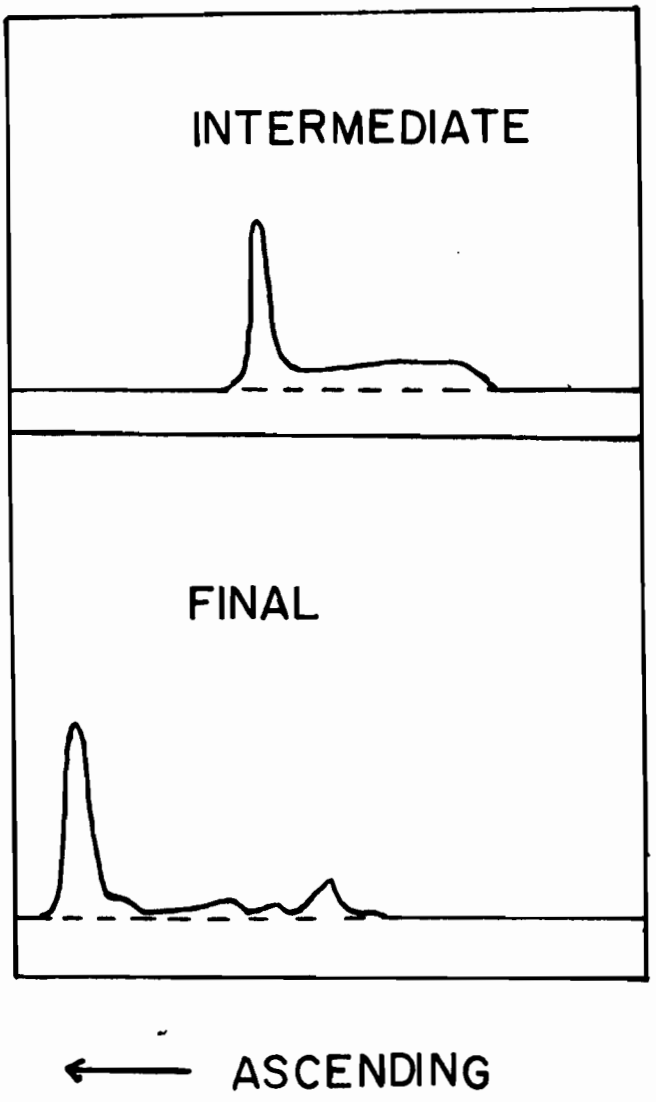
FIGURE 3

Free boundary electrophoresis of the crude ammonium pectate in sodium chloride solution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geneous, consisting of one fast-moving and at least three or four slow-moving components. Column chromatography (DEAE-Cellulose) (Fig. 4) gave three fractions having the compositions shown in Table 6. From the results it is clear that the crude ammonium pectate contained a considerable quantity of neutral polysaccharides. This was also indicated by the behaviour of the material on electrophoresis by the convection technique to be described later. The presence of an araban or a galactan could not be proved or disproved.

Purification

The ammonium pectate, after being dissolved in water, was treated with calcium chloride solution to precipitate calcium pectate which was washed successively with water, 8% aqueous sodium hydroxide, dilute acetic acid and water. Treatment of the calcium pectate with 0.5% ammonium oxalate solution furnished the purified ammonium pectate in a yield of about 30% of the crude product. It had $[\alpha]_D$ +230° in water and contained D-galacturonic acid, D-galactose and L-arabinose in a ratio of 85:4:11, as well as a trace of L-rhamnose. Free boundary electrophoresis of the purified product (Fig. 5) indicated that it was non-uniform. It is possible that during the process of purification the neutral polysaccharides were incompletely removed due to their co-precipitation with the calcium pectate. Attempts were accordingly made to resolve the purified ammonium pectate by further fractionations.

Attempted Fractionations of the Purified Ammonium Pectate

Several attempts were made to fractionate the purified ammonium pectate further by reprecipitation with calcium chloride, precipitation

FIGURE 4

Chromatography of crude pectin on DEAE-Cellulose

(phosphate form)

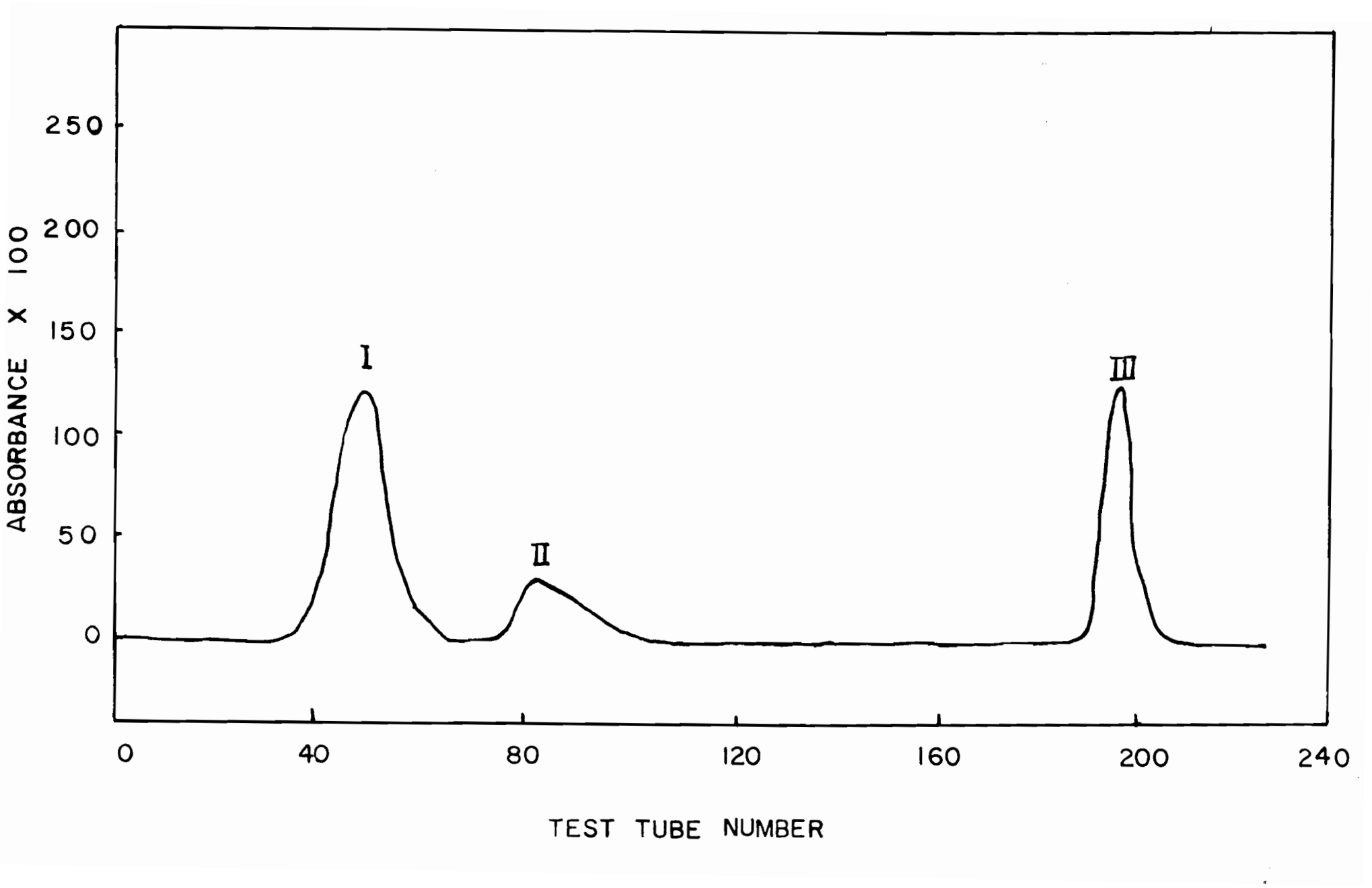

ABSORBANCE X 100
250
200
150
100
50
0
I
II
III
0
40
80
120
160
200
240
TEST TUBE NUMBER

TABLE 6

Chromatography of crude ammonium pectate on DEAE-Cellulose (phosphate form)

Eluate	% of crude pectin	Sugar Residues						
		Uronic acid	Gal	Glu	Man	Ara	Xyl	Rha
I (water)	52	-	+	++	+++	-	-	-
II (phosphate buffer)	7	+	trace	+	-	+	++	-
III (sodium hydroxide)	28	+++	+	-	-	++	trace	trace

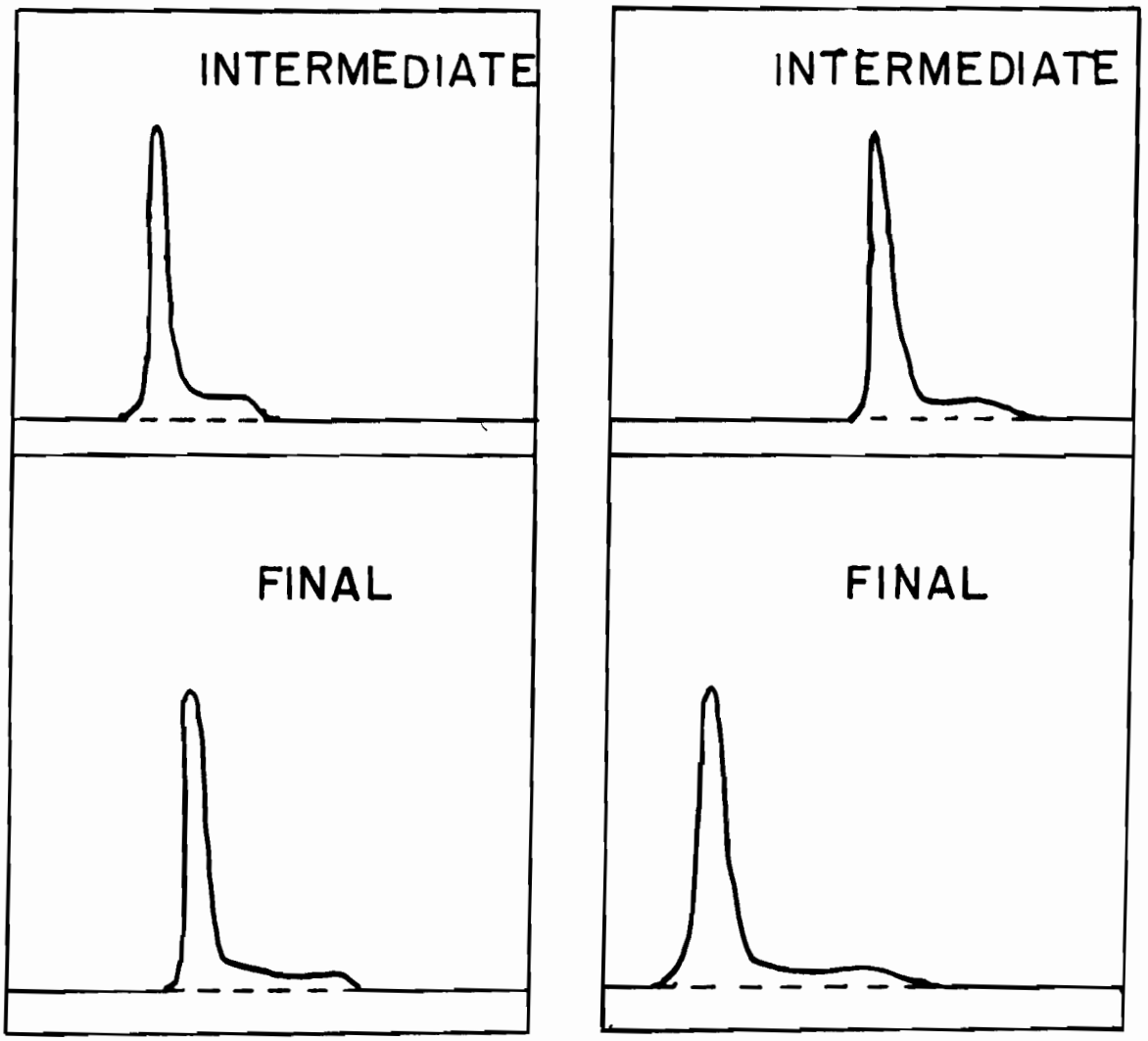
\- Absence of the corresponding sugar.

+, ++, +++ Presence of the corresponding sugar in an increasing amount.

The same notations are used to present qualitative results in later Tables.

FIGURE 5

Free boundary electrophoresis of purified ammonium pectate in borate solution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FINAL
FINAL
→ DESCENDING
← ASCENDING

with cetyltrimethylammonium hydroxide, fractional precipitation with ethanol, ion exchange chromatography on diethylaminoethylcellulose (phosphate form) and electrophoresis, but in each case the regenerated polysaccharide was virtually unchanged in composition and optical rotation as shown in Table 7.

The polysaccharide was subjected to chromatography on diethylaminoethylcellulose by the procedure of Neukom, Deuel, Heri and Kündig (53). No material could be obtained from the water or phosphate eluates which suggested the absence of araban, galactan or any other neutral polysaccharide in the purified ammonium pectate (53,54). The polysaccharide was eluted in a single band with increasing concentrations of sodium hydroxide (Fig. 6).

Yean and Goring (55) showed that sodium lignin sulfonate could be conveniently and satisfactorily separated from hemicellulose by the electrophoresis-convection technique. The efficiency of the separation was demonstrated quantitatively on a mixture of two parts of methyl cellulose and one part of a carbohydrate-free lignin sulfonate. When this technique was applied to the crude ammonium pectate, it was possible to isolate few fractions with different sugar compositions. The results are presented in Table 8. Although the collection of the fractions was rather arbitrary, it is clear that the polysaccharides obtained from top layers (fractions I, II and III) were rich in neutral sugars, whereas the lowest layer (fraction IV), which became viscous and turbid in the course of the electrophoresis, was rich in galacturonic acid. Electrophoresis of the purified ammonium pectate under identical conditions did not give any polysaccharide from the top layers and only one fraction rich in galacturonic acid could be isolated from the lowest layer. Further

TABLE 7

Attempted fractionations of the purified ammonium pectate

Fractionation methods	Yield, % of the original material	$[\alpha]_D$ degrees, (c, 1.0 in water)	Sugar Residues			
			GalA	Gal	Ara	Rha
1. Precipitation with calcium chloride (twice)	88	+235	85.0	5.0	10.0	trace
2. Precipitation with cetyltrimethyl-ammonium hydroxide	90	+232	83.8	4.5	11.7	trace
3. Chromatography on DEAE-Cellulose (phosphate form)	84	+235	85.0	5.2	9.8	trace
4. Fractional pptn. with ethanol						
i) 1st fraction	40	+230	85.1	4.9	10.0	trace
ii) 2nd fraction	30	+228	83.5	5.0	11.5	trace
iii) 3rd fraction	24	+235	85.0	4.0	11.0	trace
5. Electrophoresis (convection technique)	85	+230	84.0	5.5	10.5	trace

FIGURE 6

Chromatography of the purified ammonium pectate on DEAE-Cellulose (phosphate form)

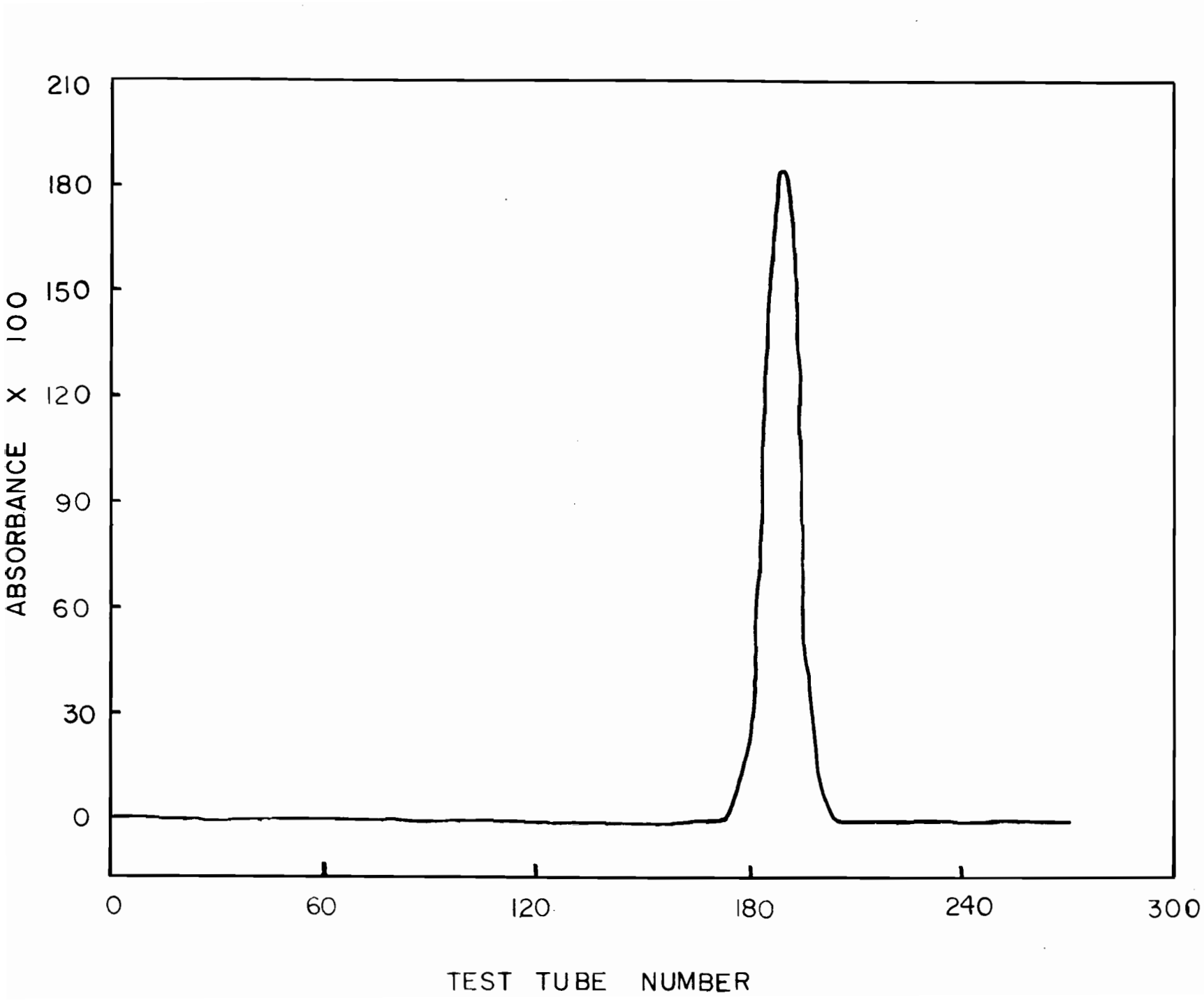

210
180
150
120
90
60
30
0
ABSORBANCE X 100
0
60
120
180
240
300
TEST TUBE NUMBER

TABLE 8

Fractions obtained on electrophoresis (convection technique) of crude ammonium pectate

Fraction	Yield, % of crude material	$[\alpha]_D$ in water, degrees	Sugar Residues						
			GalA	Gal	Glu	Man	Ara	Xyl	Rha
I	10	+80	trace	+	+++	++	+	+	-
II	32.4	+85	+	+	+++	+++	+	+	trace
III	13	+130	+	++	+	+	++	+	+
IV	12.2	+225	+++	+	-	-	++	trace	+

treatment of the turbid, viscous layer obtained in both cases and its significance are described in the next section. This experiment shows the usefulness of this technique for the separation of acidic and neutral polysaccharides.

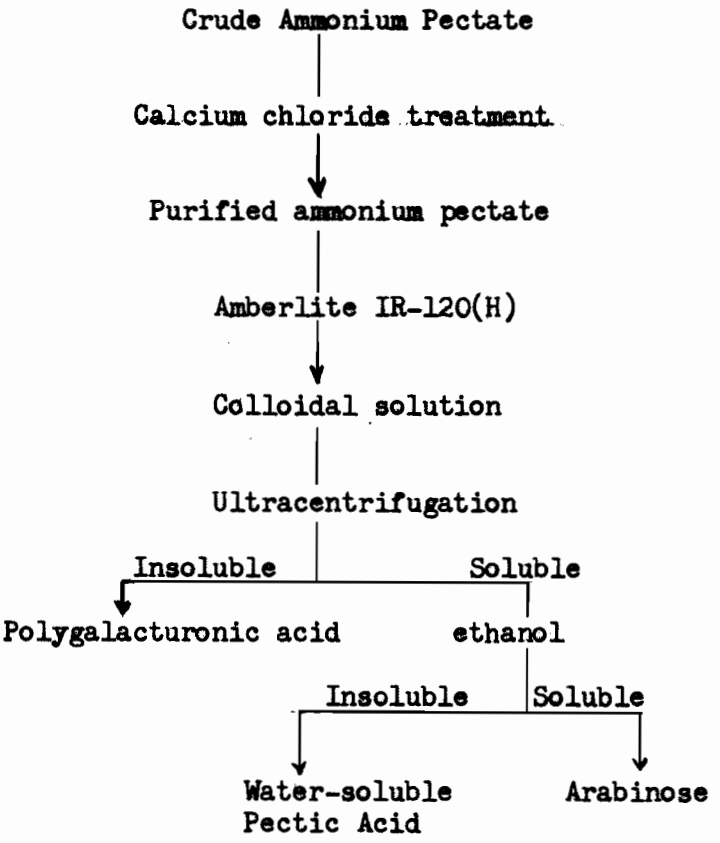
Electrophoresis on glass fiber paper with different buffer systems was attempted but no fractionation could be achieved.

Fractionation of the Purified Ammonium Pectate

The results of the various fractionation experiments suggested that the purified ammonium pectate must be free from neutral polysaccharides. Although acidic polysaccharides differing in their acidic character should be resolvable by chromatography on DEAE-Cellulose (56), the possibility of non-homogeneity of the purified ammonium pectate could not be excluded. It could indeed be fractionated into two parts by several different methods as described below on the basis of the difference in solubility of the free acids in water.

When the aqueous solution of the purified ammonium pectate was passed through Amberlite IR-120(H) exchange resin, a colloidal solution was obtained. After concentration to a smaller volume, the eluate was subjected to ultracentrifugation at 20,000 r.p.m. (40210 g) and the supernatant solution was precipitated with ethanol to give a water-soluble pectic acid containing galacturonic acid, galactose, arabinose and rhamnose. The solid residue was washed with water several times and recovered by ultracentrifugation each time. Finally a water-insoluble product was obtained consisting only of galacturonic acid residues. The infrared spectra of the unfractionated ammonium pectate, the water-insoluble polygalacturonic acid and water-soluble pectic acid (Fig. 12) revealed the

completeness of the ion exchange treatment. Other aspects of the infrared spectra of these materials will be discussed later. A small quantity of arabinose could be recovered from the filtrate after removal of the precipitate of the water-soluble pectic acid, as shown in the flow-sheet below.



It is possible that the exchange resin was acidic enough and also accessible enough to effect removal of some of the acid-labile arabinofuranose.

Similar fractions could be obtained by acidification of the aqueous solution of the purified ammonium pectate with N-sulfuric acid, followed by ultracentrifugation.

As stated before, a colloidal viscous layer was obtained by the

electrophoresis-convection technique from both the crude and purified ammonium pectates. In both cases a part of this fraction was diluted with water and then subjected to ultracentrifugation when two fractions (water-insoluble and water-soluble) were obtained.

Fractionation of polysaccharides using salts such as ammonium sulfate or potassium chloride is sometimes quite efficient. Potassium chloride had been used successfully for the fractionation of alginic acid (57). By the addition of an aqueous solution of sodium chloride to the water-solution of the ammonium pectate it was possible to obtain two similar fractions. Of course, in this case the polysaccharides were recovered as salts.

The water-insoluble and water-soluble portions, respectively, obtained by the different techniques mentioned above were identical as far as their specific rotations and sugar composition were concerned (Table 9). It is evident that the water-insoluble part was free from neutral sugars. The detection of some free L-arabinose in the eluate after treatment of the unfractionated ammonium pectate with Amberlite IR-120(H), as mentioned before, indicates that some arabinofuranose residues could be originally linked to the water-insoluble polygalacturonic acid. However, the isolation of a polygalacturonic acid devoid of any neutral sugar residue by the application of the electrophoresis-convection technique to the crude and purified ammonium pectates, where such a cleavage was not possible, eliminated this possibility.

Enzymic Hydrolysis

When the unfractionated ammonium pectate was treated with pectinase, digalacturonic acid and trigalacturonic acid were obtained.

TABLE 9

Water-insoluble and water-soluble portions obtained by different methods

Method of Fractionation	Water-insoluble portion			Water-soluble portion				
	Yield, % of the unfractionated material	$[\alpha]_D$(a) in water, degrees	Components	Yield, % of the unfractionated material	$[\alpha]_D$ in water, degrees	Sugar composition(b)		
						GalA	Gal	Ara
1. Treatment with Amberlite IR-120(H)	50	+246	Galacturonic acid	30	+220	74.4	7.1	18.5
2. Acidification with N sulfuric acid	54	+240	Galacturonic acid	28	+225	74.2	8.0	17.8
3. Electrophoresis-convection	48	+239	Galacturonic acid	32	+222	73.0	8.0	19.0
4. Treatment with aqueous sodium chloride	58	+236	Galacturonic acid + arabinose (trace)	31	+219	74.0	7.5	18.5

(a) $[\alpha]_D$ values reported were of the Na-salt of the polygalacturonic acid except in (4).

(b) A minor amount of rhamnose was present in all four cases.

The identification of these oligosaccharides and other results are described later.

Partial Acid Hydrolysis of the Crude Ammonium Pectate

The crude ammonium pectate was partially hydrolyzed with N-sulfuric acid, giving small quantities of oligosaccharides, as shown in Table 10. Only a preliminary study of these could be made at this stage. As will be seen later, a partial hydrolyzate of the purified ammonium pectate contained all these oligomers except one containing galacturonic acid and galactose.

TABLE 10

Acidic oligosaccharides obtained on partial acid hydrolysis of the crude ammonium pectate

Yield, % of the crude product	R_{GalA} value in solvent D	$[\alpha]_D$ in water, degrees	Component sugars on hydrolysis	Neutralization equivalent
(a) 0.50	0.05	+184	Galacturonic acid	
(b) 0.25	0.10	+ 79	Galacturonic acid, rhamnose	505
(c) 0.06	0.15		Galacturonic acid, galactose	
(d) 0.65	0.19	+145	Galacturonic acid	
(e) 0.31	0.81	+ 75	Galacturonic acid, rhamnose	350

PART II

The Polygalacturonic Acid

The water-insoluble polymer gave only galacturonic acid on complete hydrolysis. The sodium salt of this polygalacturonic acid had $[\alpha]_D$ +246° in water and was homogeneous as suggested by free boundary electrophoresis (Fig. 7).

Enzymic Hydrolysis

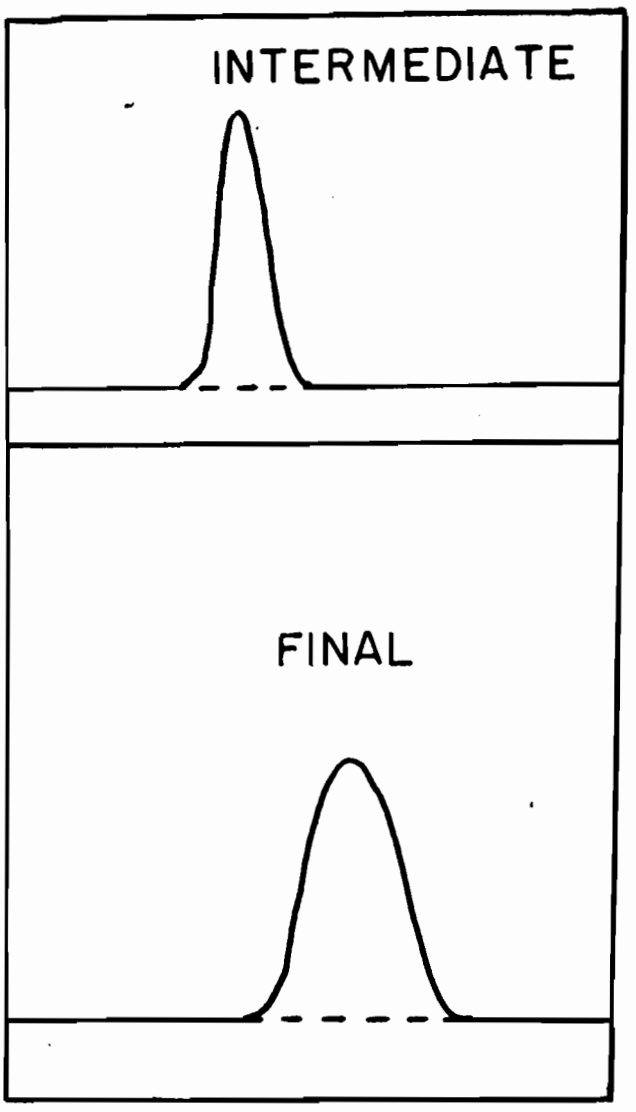
It is well-known that pectinase can degrade pectin to yield oligomers, but in order to get better yields, as pointed out by Kuhn (58), the oligomers formed should be protected from further degradation. A suitable technique for this purpose was suggested by Painter (59), and applied successfully by Perilä and Bishop (60) and also by Timell (61). The same technique, using continuous dialysis, was used in the present case.

A commercial preparation of fungal origin, designated as "pectinase" and probably constituting a mixture of several enzymes was used. It has been described as highly unspecific (61). An aqueous solution of sodium polygalacturonate was employed for the enzymic hydrolysis. The dialyzate which contained different oligouronides and galacturonic acid, was concentrated, treated with Amberlite IR-120(H) and fractionated by paper chromatography.

It was originally shown by French (62) that if $\log R_f/(1-R_f)$ is plotted against n, where R_f is the rate of movement of a sugar on the paper chromatogram relative to the solvent front and n is the number of residues in an oligosaccharide, straight lines should be obtained, different for different polymer-homologous series of sugars. This relation-

FIGURE 7

Free boundary electrophoresis of polygalacturonic acid in borate solution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ship seemed to be valid even when R_f is replaced by Rx, where Rx is the relative movement with respect to a particular monomer instead of the solvent front (61,63).

When log $R_{GalA}/(1 - R_{GalA})$ values for different oligouronides obtained by the enzymic hydrolysis of the polygalacturonic acid were plotted against n, where R_{GalA} is the rate of movement of oligomer relative to galacturonic acid in solvent B, a linear relationship was obtained, as shown in Fig. 8 suggesting that the oligouronides belonged to a homologous series. The dimer and trimer were shown to be α-(1 --> 4)-linked D-galacturonides, as will be described later.

Methylation Studies

Methylation is one of the most useful techniques available in the structural investigation of polysaccharides. In most cases partial methylation of the material is initially achieved by using dimethyl sulfate and sodium hydroxide (68). Recently, a method using barium oxide and methyl iodide in dimethyl sulfoxide has been used for the initial partial methylation of several polysaccharides (69,70), but in view of the insolubility of barium pectate this is probably not a very suitable procedure for partial methylation of pectin. Due to the instability of the galacturonosidic linkages towards alkali (64,65,66,97) it is also not desirable to methylate pectin with dimethyl sulfate and sodium hydroxide without proper consideration of this factor. The instability of esters of pectic acid has been explained by Neukom and Deuel (66) by assuming a β-alkoxy elimination (Fig. 9), as suggested by Kenner (67) for the breakdown of oligo- and polysaccharides. The carboxylate group at C-6 is not sufficiently electronegative to activate the hydrogen at

FIGURE 8

Relationship of $\log \left[R_{GalA} / (1 - R_{GalA}) \right]$ with n (degree of polymerization) of oligomers

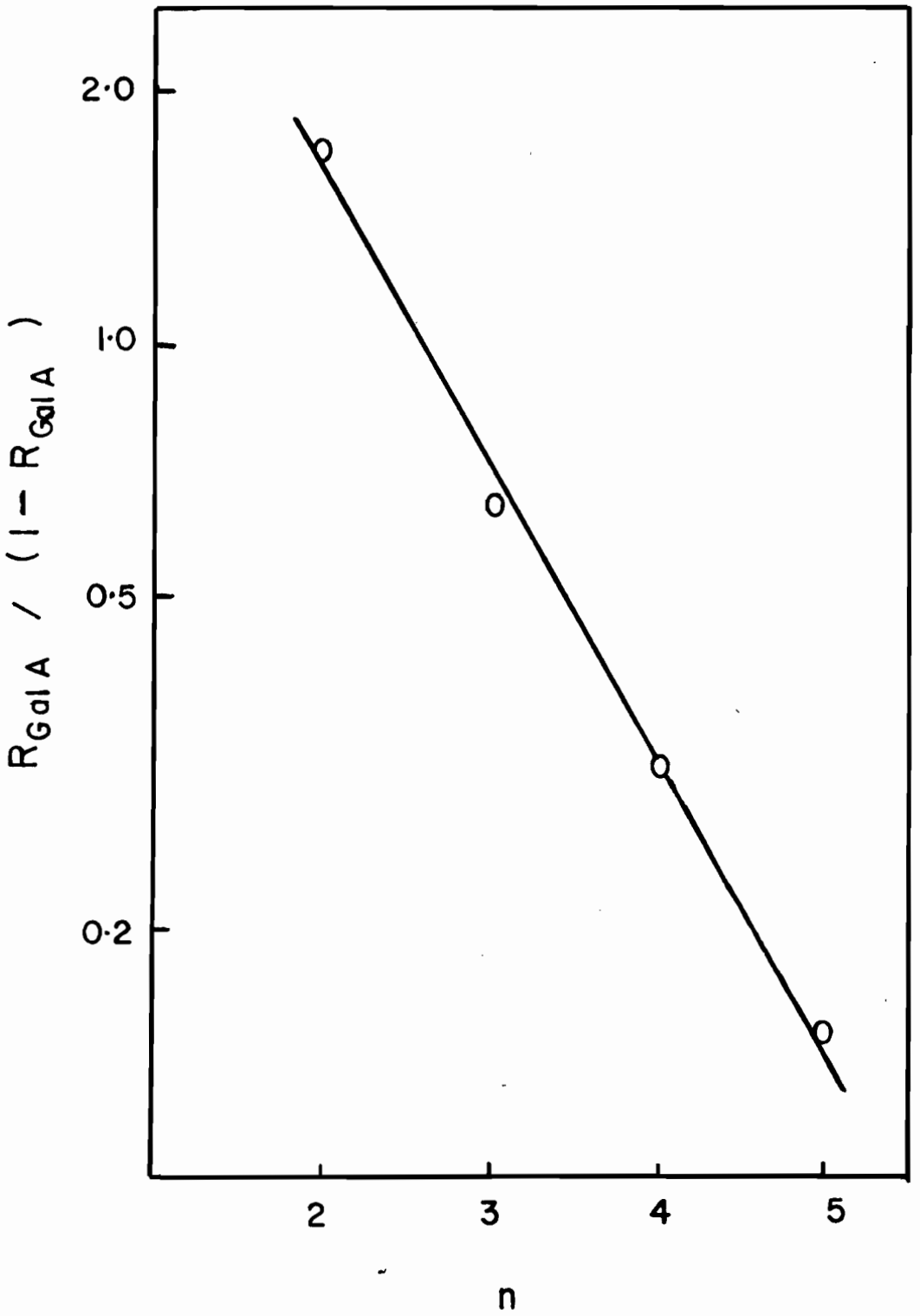
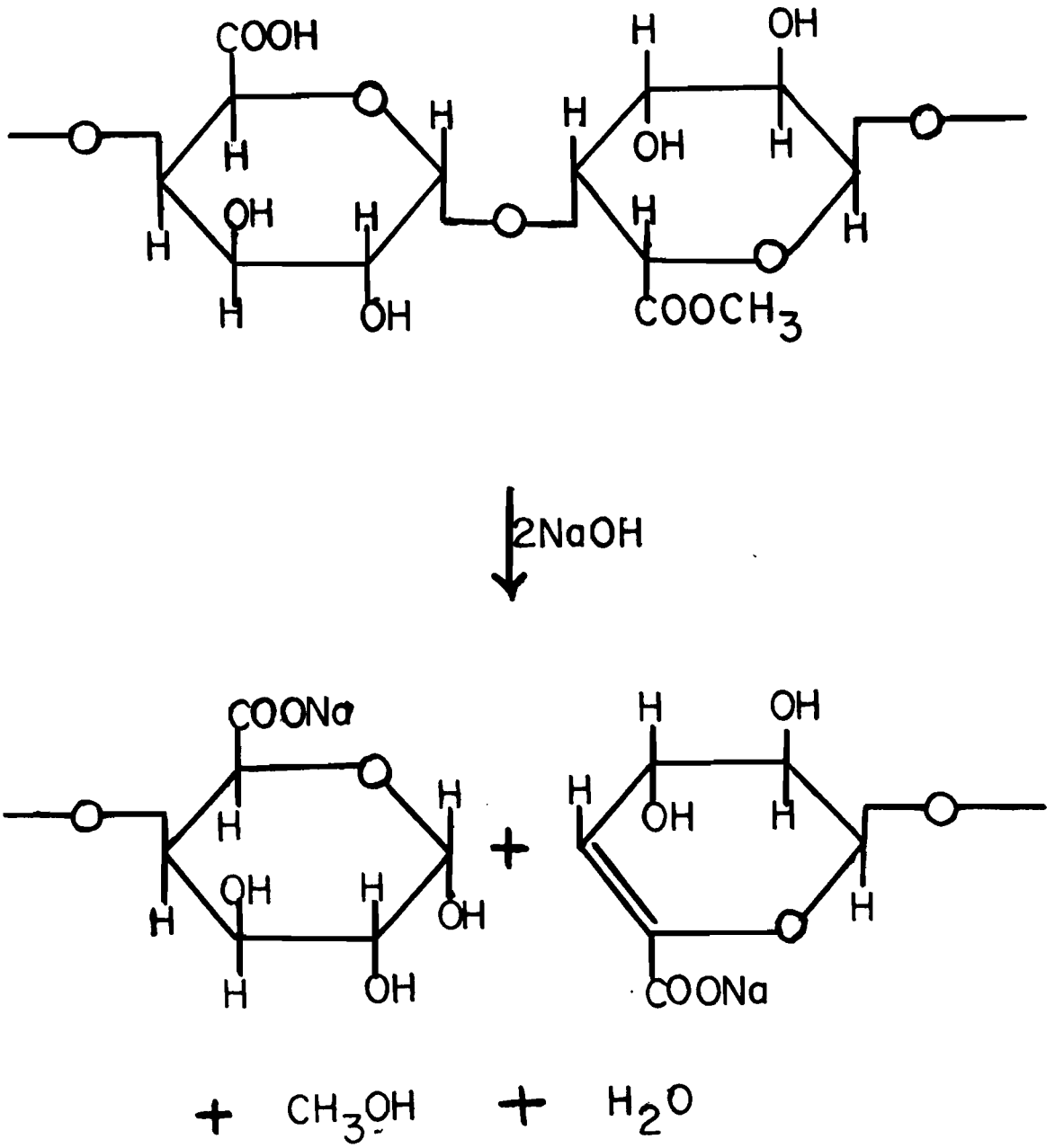

2·0
1·0
0·5
0·2
$R_{GalA} / (1 - R_{GalA})$
2
3
4
5
n

FIGURE 9

Alkaline degradation of pectin


COOH
H
OH
H
H
OH
H
H
OH
H
H
OH
H
H
COOCH3
2NaOH
COONa
H
OH
H
H
OH
H
H
OH
H
H
OH
H
H
COONa
+
+ CH3OH + H2O

C-5 in alkali and the cleavage of the (1 --> 4) glycosidic bond does not, therefore, generally occur under these conditions. Although the polyglycosiduronic acids are more stable than their esters, they are not completely stable in alkaline solution (71). It is possible to adopt either of the following two procedures in order to minimize degradation during methylation. Either the polyuronide should be methylated for a limited period of time with dimethyl sulfate and sodium hydroxide and then be further methylated by the method of Kuhn (72), or the carboxyl group of uronic acid residues should be reduced. In the present case the latter alternative was adopted. The reduced polyuronide can be obtained either by acetylation of the product followed by reduction of the free acid with diborane (73,74), or by esterification with propylene oxide or ethylene oxide (75) followed by reduction of the ester with sodium borohydride. It is often necessary to repeat the esterification and reduction cycle several times to achieve complete reduction. It has been reported that the esterification of alginic acid with propylene oxide proceeded better in organic solvents than in water, although the initial rate in water was higher than in some of the organic solvents (76).

In the present case the polygalacturonic acid was treated with propylene oxide to form the glycol ester which was subsequently reduced with sodium borohydride. The esterification-reduction cycle was repeated four times when galactose and galacturonic acid were present in a ratio of 94:6. The reduced product was used for methylation. An attempt to reduce the polygalacturonic acid, after prior acetylation (77), by using diborane was made later, but complete reduction was not achieved, probably because of the difficulty in dissolving the acetylated product in diglyme,

which was used as a solvent during the reduction.

The reduced product was methylated first with dimethyl sulfate and subsequently with methyl iodide and silver oxide to yield a methylated galactan with a methoxyl content of 42.6% and $[\alpha]_D$ +125° in chloroform. The methoxyl content could not be raised by further methylation.

A portion of the methylated product was refluxed with N-methanolic hydrogen chloride and then hydrolyzed with N-sulfuric acid. The O-methyl sugars were separated by paper chromatography. The relative amounts are presented in Table 11.

The major fraction (b) was identified as 2,3,6-tri-O-methyl-D-galactose by its conversion to the corresponding galactonolactone. The minor fraction (a) was 2,3,4,6-tetra-O-methyl-D-galactose characterized as its aniline derivative. The third fraction (c) was mainly 2,3-di-O-methyl-D-galacturonic acid. The presence of this compound was clearly due to incomplete reduction of the polygalacturonic acid. After reduction of the carboxyl group with lithium aluminum hydride this fraction was chromatographically homogeneous and consisted of 2,3-di-O-methyl-D-galactose, identified through its aniline derivative. It is evident that 2,3,6-tri-O-methyl-D-galactose and 2,3-di-O-methyl-D-galacturonic acid formed the major part of the mixture of methylated sugars. These compounds could arise only from (1 --> 4)-linked D-galacturonic acid residues present in the original, acidic polysaccharide. It was possible to isolate only few crystals (ca. 3 mg.) of the aniline derivative of fraction (a), indicating that this fraction might have been contaminated by some unidentified impurities. Therefore, the isolated amount (10 mg.) should not be used for obtaining the molar ratio of the components. The presence of impurities in this fraction was also indicated by thin layer chromato-

TABLE 11

Methylated sugars obtained on hydrolysis of the methylated polygalactan

Fraction	R_G value in solvent G	R_G value in solvent H	Weight, mg.	Identity	Mole per cent
(a)	0.90	0.80	10(?)	2,3,4,6-tetra-O-methyl-D-galactose	0.9
(b)	0.73	0.50	980	2,3,6-tri-O-methyl-D-galactose	95.2
(c)		0.00	41	2,3-di-O-methyl-D-galacturonic acid	3.9

graphy on silica gel-G and also a model experiment in which 2,3,4,6-tetra-O-methyl-D-galactose was synthesized and converted to its aniline derivative on different scales (including a 10 mg. scale). The yield of the anilide was found to be 110% of 2,3,4,6-tetra-O-methyl-D-galactose. If the molar ratio of 2,3,4,6-tetra-O-methyl-D-galactose, as shown in Table 11 and the number average degree of polymerization of 450, obtained by osmometric measurements, are taken into consideration, it appears that the polygalacturonic acid might be slightly branched, a situation which is quite unlikely for two reasons. Firstly, if there was branching at positions 2 or 3 there should be some 2,6-di-, 3,6-di- or some mono-O-methyl-D-galactoses which were not obtained in the present case. Secondly, this type of branching should give some (1 --> 2)- or (1 --> 3)-linked oligouronides on partial hydrolysis of the polysaccharide, compounds which were not observed. It seems reasonable, therefore, to assume that the aniline derivative was formed in a yield of 110%, which would give 2.7 mg. of the tetra-O-methyl-D-galactose, resulting in a molar ratio of 0.20, 96.0 and 3.9 for fractions (a), (b) and (c) respectively (Table 11).

As it was not possible to reduce the carboxyl groups completely with sodium borohydride, the remaining uronic acid portion would give methylated sugar acids on hydrolysis of the methylated polysaccharide. A small sample of the methylated polysaccharide was, therefore, reduced with $LiAlH_4$ in tetrahydrofuran, methylated again with silver oxide and methyl iodide and then hydrolyzed. In addition to the main fraction of 2,3,6-tri-O-methyl-D-galactose and the minor fraction of 2,3,4,6-tetra-O-methyl-D-galactose, another small fraction having the same mobility of 2,3-di-O-methyl-D-galactose was now obtained.

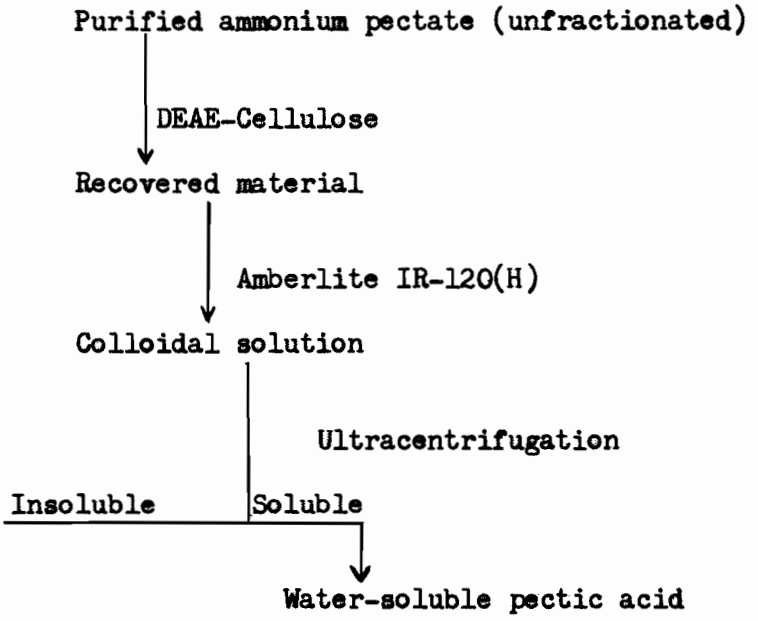
All these results suggested that the water-insoluble polygalact-

uronic acid was a straight-chain polymer consisting of (1 --> 4)-linked α-D-galactopyranosyluronic acid residues. This represents the first isolation from bark of a true polygalacturonic acid, completely free from neutral sugars. Its high degree of polymerization, unbranched character, and low methoxyl content (calculated for completely esterified polygalacturonic acid: OMe, 16.3%; found: OMe, 0.6%, compared to a methoxyl value of 2.7% for the water-soluble pectic acid) could probably explain its solubility characteristics (93).

PART III

The Water-Soluble Pectic Acid

Unlike the polygalacturonic acid, the water-soluble polysaccharide contained D-galacturonic acid, D-galactose, L-arabinose and L-rhamnose in a ratio of 73:7:18:2. Free boundary electrophoresis of this water-soluble pectic acid (Fig. 10) suggested that it was not homogeneous. A similar electrophoresis pattern (Fig. 11) was obtained with a sample of water-soluble pectic acid obtained in a slightly modified way, as shown in the flow-sheet below.



On the other hand, chromatography on DEAE-Cellulose (phosphate) using gradient elution with sodium hydroxide showed a single peak. The product obtained from the DEAE-Cellulose column and the materials resulting after treatment with calcium chloride and cetyltrimethylammonium hydroxide had similar compositions, as shown in Table 12. All these results suggested that it might be a mixture of very closely related acidic polysaccharides

FIGURE 10

Free boundary electrophoresis of the water-soluble pectic acid in borate solution

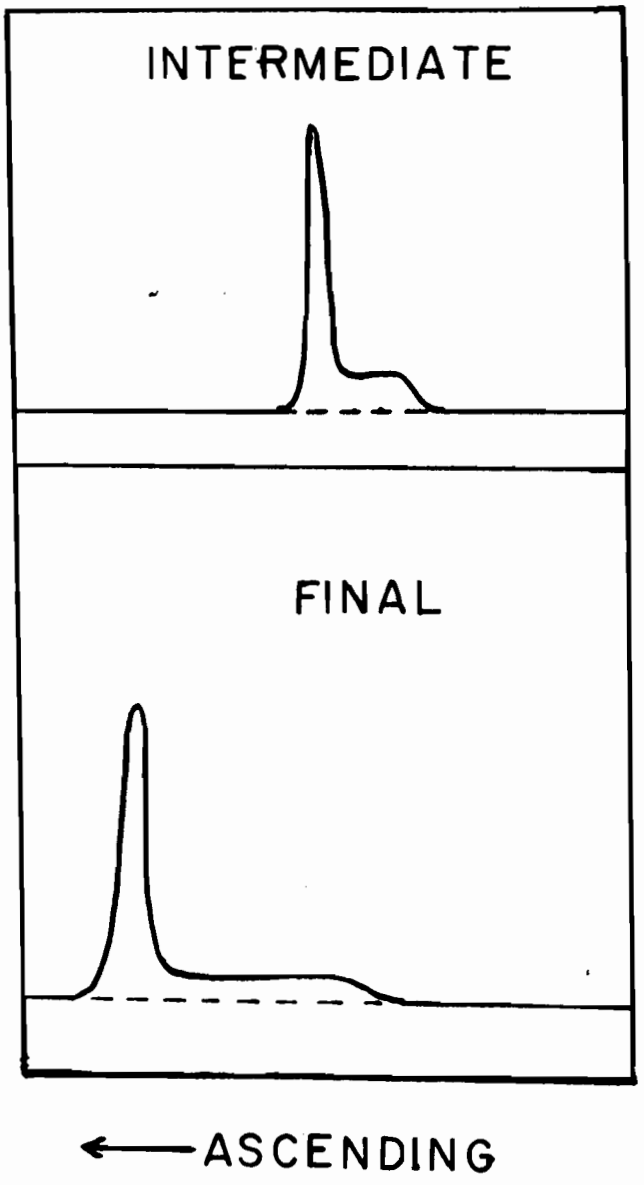

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FIGURE 11

Free boundary electrophoresis of the water-soluble pectic acid, obtained after chromatography of unfractionated material on DEAE-Cellulose (phosphate), in borate solution

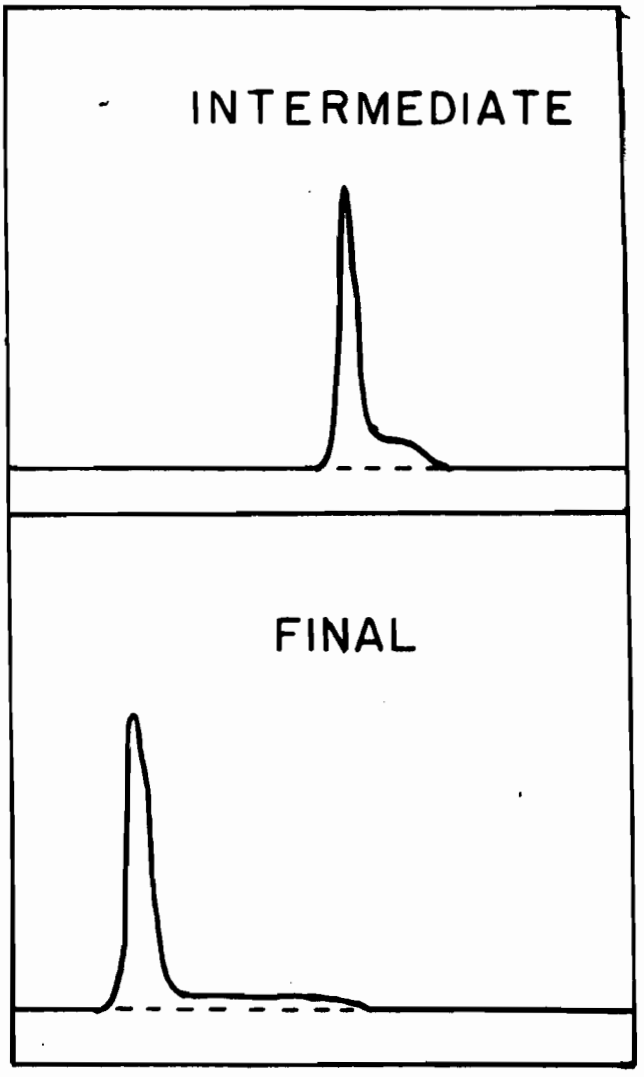
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TABLE 12

Attempted fractionations of water-soluble pectic acid

Method	Yield, % of the original material	$[\alpha]_D$, in water, degrees	Sugar residues[(a)] in relative per cent		
			GalA	Gal	Ara
1. Repeated precipitation with calcium chloride	90	+222	74.5	7.0	18.5
2. Precipitation with cetyltrimethylammonium hydroxide	92	+225	75.0	7.5	17.5
3. Chromatography on DEAE-Cellulose	80	+216	74.0	7.5	18.5
4. Fractional precipitation with ethanol					
i)	30	+222	74.0	8.0	18.0
ii)	35	+218	74.4	8.2	17.4
iii)	20	+220	74.0	7.8	18.2

(a) Rhamnose (in minor amount) was present in all fractions almost to the same extent which was not estimated.

differing slightly in their content of galacturonic acid residues and in molecular size. Gel filtration using Sephadex G-200 failed to resolve it further, which probably indicates the absence of very low molecular-weight chains. The results obtained on chromatographic resolution suggested (56) the absence of neutral or slightly acidic araban or a galactan (38,39).

It was evident that the neutral sugars were probably integral parts of the pectic acid and it was accordingly decided to subject the polysaccharide to a structural investigation. Different possibilities existed for the mode of attachment of the neutral sugars. The neutral sugars might be located in the main chain of a polygalacturonic acid or they might be directly attached to the main chain as single unit side chains. Alternatively, the neutral sugar residues could be attached to the end of different branches or form short chains linked directly to the acid-framework. From the results obtained on methylation and on partial hydrolysis it was possible to exclude some of the possibilities mentioned above.

Methylation of the Water-soluble Pectic Acid

As stated before, it is necessary to reduce the carboxyl group of pectic acid before subjecting it to the action of dimethyl sulfate and sodium hydroxide in order to minimize alkaline degradation. In the present case, with a pectic acid containing galactose, arabinose and rhamnose in addition to galacturonic acid, a prior reduction would make it impossible to obtain any information concerning the linkage of the galactose residues. The pectic acid was, therefore, methylated without prior reduction. Dimethyl sulfate and alkali were used a minimum number

of times, followed by methylation to completion with methyl iodide and silver oxide. The methylated methyl pectate (OMe, 39.2%) was hydrolyzed with N-sulfuric acid and resolved into an acidic and a neutral portion using anion exchange resin. The acidic fraction after being treated with anhydrous methanolic hydrogen chloride was reduced with lithium aluminum hydride and hydrolyzed. The O-methyl sugars thus obtained were separated by preparative paper chromatography (Table 13). Fraction A4 was further resolved into two components using paper chromatography. The neutral portion was resolved into six fractions using paper chromatography. The amount and characteristics of all fractions are presented in Table 14.

The methylated sugars (fractions A1 to A5 as shown in Table 13) originated from galacturonic acid and the components N1 to N5 (Table 14) from the neutral sugars present in the original polysaccharide. Fraction A1 contained only one component, 2,3,4-tri-O-methyl-D-galactose, identified by its conversion to the crystalline aniline derivative. Fraction A2 was identified as 2,3-di-O-methyl-D-galactose by its conversion to crystalline aniline derivative. Component A3 was obtained in a very small quantity and was probably an artifact. The crystalline fraction A4(ii) was identified as 2-O-methyl-D-galactose. Component A4(i) was tentatively identified as 3-O-methyl-D-galactose. Among the neutral fractions N1 was characterized as 2,3,5-tri-O-methyl-L-arabinose by its conversion to the crystalline 2,3,5-tri-O-methyl-L-arabonamide. Another major fraction, N2, was identified as 2,3,4,6-tetra-O-methyl-D-galactose through its crystalline aniline derivative. Components N3 and N4 were tentatively characterized as a di-O-methyl-L-arabinose and 2,3,4-tri-O-methyl-D-galactose respectively. Since no 2,3,6-tri-O-methyl-D-

TABLE 13

Methylated sugars obtained from the acidic portion of the hydrolyzate of methylated methyl pectate

Fraction	Weight, mg.	$[\alpha]_D$ in water, degrees	Sugar on demethylation	R_G values in solvent H	R_G values in solvent G
A1	11	+107	galactose	0.36	0.66
A2	336	+ 78	galactose	0.21	0.51
A3	2		galactose	0.11	0.40
A4	140	+ 85	galactose	0.04	0.29
A5	28			0.00	0.16

Resolution of A4

Components	Relative percentage of A4	$[\alpha]_D$ in water, degrees	$R_{galactose}$ values in solvent A	R_G values in solvent G	M_G values
A4(i)	48	+101	2.0	0.24	0.72
A4(ii)	52	+ 80	2.35	0.31	0.44

TABLE 14

Methylated sugars obtained from the neutral portion of the hydrolyzate of methylated methyl pectate

Fraction	Weight, mg	$[\alpha]_D$ in water, degrees	Sugar on demethylation	M_G values	R_G values solvent H	R_G values solvent G
N1	60	-36	arabinose	0.00	1.08	0.92
N2	40	+106	galactose	0.00	0.80	0.87
N3	10	+ 65	arabinose + trace of xylose	0.00	0.48	0.63
N4	6		galactose	0.00	0.40	0.72
N5	11	+ 40	rhamnose	0.37	0.24	0.61
N6	8		galactose: arabinose: rhamnose in an approx. ratio of (3:4:1)			

galactose was formed it follows that (1 --> 4)-linked galactose residues were absent. Fraction N5 was characterized as 3-O-methyl-L-rhamnose while fraction N6 was a mixture of different sugars, probably mono-methylated galactose, arabinose and some unmethylated rhamnose.

Taking into consideration the relative amount and the composition of the acidic and the neutral portions, the composition of the entire mixture of O-methylated sugars was obtained. The data are presented in Table 15. The structural significance of these results may be assessed by considering the various acidic and neutral components. It is clear that the main chain of the polysaccharide consisted of D-galacturonic acid residues linked together by (1 --> 4)-glycosidic bonds. The high positive rotation suggested that these residues were present in the α-modification. The amount of 2-O-methyl-D-galacturonic acid, 3-O-methyl-D-galacturonic acid and also D-galacturonic acid suggested that these constituted branching points in the polymer. Since D-galactose and L-arabinose appeared mainly as the 2,3,4,6-tetra-O-methyl- and 2,3,5-tri-O-methyl-derivatives, respectively, it can be said with certainty that most of the D-galactose and L-arabinose residues were present as non-reducing end groups. At this stage it was not clear whether they were directly attached to the 2 or the 3-position of galacturonic acid within the chain or attached to some branches of D-galacturonic acid. This point is discussed later in the light of the results obtained on partial hydrolysis. The small quantities of 2,3,4-tri-O-methyl-D-galactose and di-O-methyl-L-arabinose left some ambiguity as to their origin. These compounds could be the result of incomplete methylation or demethylation during hydrolysis of the methylated polysaccharide. Alternatively there might be some L-arabinose and

TABLE 15

Relative molar ratio of different methylated sugars originating from water-soluble pectic acid

Component	Mole per cent
2,3,4-Tri-O-methyl-D-galacturonic acid	1.4
2,3-Di-O-methyl-D-galacturonic acid	45.3
Di-O-methyl-D-galacturonic acid	0.3
3-O-Methyl-D-galacturonic acid	9.7
2-O-Methyl-D-galacturonic acid	10.5
D-Galacturonic acid	4.3
2,3,5-Tri-O-methyl-L-arabinose	13.3
2,3,4,6-Tetra-O-methyl-D-galactose	7.2
Di-O-methyl-L-arabinose	2.4
2,3,4-Tri-O-methyl-D-galactose	1.2
3-O-Methyl-L-rhamnose	2.6
Mixture	1.8

(1 --> 6)-linked D-galactose residues located within the main chain or in branches. Most of the L-rhamnose residues of the polysaccharide were obtained as 3-O-methyl-L-rhamnose. No other methylated rhamnose could be detected. Although trace amounts of 3,4-di-O-methyl-L-rhamnose might have occurred along with the 2,3,4,6-tetra-O-methyl-D-galactose, because of their close R_G values, the results on paper electrophoresis, where they have widely different mobilities (96), eliminated this possibility in the present case. This suggested that the rhamnose was not present as a non-reducing end group. More information concerning the mode of attachment of the L-rhamnose was obtained from partial hydrolysis experiments, to be described later.

Enzymic Hydrolysis

The action of pectinase on the polygalacturonic acid, as stated before, resulted in the formation of galacturonic acid, and its di-, tri-, tetra- and penta-mers. A similar treatment of the water-soluble pectic acid yielded only traces of tetra- and pentasaccharides. The enzymic hydrolyzate consisted of D-galacturonic acid, its dimer and trimer, and the neutral sugars. No acidic oligosaccharides containing neutral sugars were formed. The enzymic hydrolyzate of the unfractionated ammonium pectate showed a lower yield of the higher oligomers, as shown in Table 16. The di- and trigalacturonic acid obtained from the water-soluble pectic acid were identical with those formed on partial acid hydrolysis.

It has been pointed out by Lineweaver and co-workers (78) that commercial pectinase contains some enzymes other than pectinesterase and polygalacturonase. The polygalacturonase acts on the de-esterified

TABLE 16

Comparison of the action of pectinase on different pectin fractions

	Oligomers of galacturonic acid			
	Dimer	Trimer	Tetramer	Pentamer
Unfractionated, purified ammonium pectate	++++	++++	+	+
Polygalacturonic acid	++++	++++	++	++
Water-soluble pectic acid	++++	++++	trace	trace

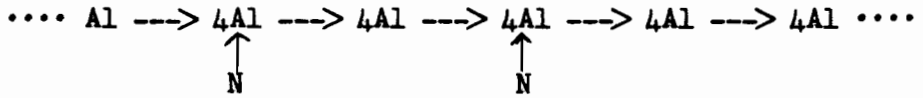
portion of the polygalacturonide chain. It was not certain, but seemed likely, that both carboxyl groups adjacent to glycosidic (1 --> 4)-linkage must be free for the linkage to be labile to polygalacturonase. It was also mentioned that if large amounts of pectinesterase are present during the enzymic hydrolysis, the rate of hydrolysis approached that of the action of polygalacturonase on pectic acid.

In the light of the existing knowledge of the nature of pectinase, it is possible to explain the results obtained here with the three different fractions of pectin. In the case of the water-insoluble polygalacturonic acid, which had practically no ester group and which was shown to have the following structure,

···· Al ---> 4Al ---> 4Al ---> 4Al ---> 4Al ---> 4Al ---> 4Al ····

A = α-D-galacturonic acid

scission might take place almost in a random fashion (although the relationship for random cleavage does not hold good exactly as can be seen from Table 17), as a result of which some higher oligouronides could be formed. In the case of the water-soluble pectic acid which was shown to have the following structural features with some side chains of neutral sugars, the cleavage of



A = α-D-galacturonic acid

N = Neutral sugars (galactose or arabinose)

the (1 --> 4)-linkage by polygalacturonase would depend on the rupture of the (N ---> A)-linkages, caused by an enzyme other than polygalacturonase

TABLE 17

Oligomers obtained from polygalacturonic acid by the action of pectinase

Compound	Amount (mg.) isolated	Total amount (mg.) (calculated) present in the hydrolyzate	Per cent of polygalacturonic acid	Maximum theoretical yield (%) for random cleavage (58)
Digalacturonic acid	56	83.0	9.3	29.8
Trigalacturonic acid	40	59.3	6.7	18.7
Tetragalacturonic acid	12	17.8	2.0	13.8
Pentagalacturonic acid	2 (approx.)	3.0	0.3	11.0

and pectinesterase. Instead of random cleavage, some selective cleavage at less hindered positions (remote from the branching points) with subsequent hydrolysis of the (N ---> A)-bonds by the other enzyme, followed by cleavage of some of the previously protected linkages would liberate only dimers and trimers of galacturonic acid. From the methylation study of the water-soluble pectic acid it was evident that there were 20-25 non-uronide side chains per 100 units of the molecule. If it is assumed that these non-uronide side chains were distributed in a random way, attached to every third or fourth galacturonic acid residue in the main chain, the possibility for the formation of higher oligomers would be small.

By the action of the same pectinase on 4-O-methylglucuronoxylan (61) oligomers of the type $X\left[-- X\right]_n-- X$ (A) were obtained first, because the pectinase was incapable of rupturing glucuronosyl bonds, and second, because steric hindrance was prominent in the immediate vicinity and to the right of the branching points. Neutral oligomers of the type $X\left[-- X\right]_n-- X$ were, however, also formed, probably because only every tenth xylose residue, on the average, carried an acid side chain. In the present case, the (N ---> A)-linkages were easily hydrolyzed by the enzyme, and higher oligomers were absent due to the large number of side chains.

Conchie and Levvy (79) have shown that the glycosidase activities of certain enzymes are inhibited in the presence of aldonolactones of the corresponding configuration. When an arabinoxylan from rye flour and cocksfoot grass were hydrolyzed by a commercial enzyme preparation in the presence of arabonolactone a series of oligosaccharides containing both xylose and arabinose was obtained, one of which was identified as O-L-

arabinofuranosyl-(1 --> 3)-O-β-D-xylopyranosyl-(1 --> 4)-D- xylose (80). Since a similar situation existed in the present case, an attempt was made to isolate some oligosaccharides containing both galacturonic acid and arabinose using pectinase in the presence of arabonolactone. However, no such oligomer could be obtained.

Partial Hydrolysis

It is known that the glycosidic bond of the (1 --> 4)-linked α-galacturonides is very resistant to acid hydrolysis compared to that of neutral polysaccharides. This property is probably due to the steric effect of the carboxyl group at C-5 and possibly also to the effect of the chain conformation (81).

In order to find suitable conditions for partial hydrolysis the water-soluble pectic acid, a preliminary study was carried out using different acid concentrations and different lengths of time. Oxalic acid (0.1N), formic acid (45%) and sulfuric acid (1N) were used and the aliquots were taken at an interval of one-half or one hour. Each aliquot was divided into two parts - the hydrolyzate and the degraded polysaccharide. The mixture of monomers and oligomers was recovered from the hydrolyzate in the usual way and the degraded pectic acid was hydrolyzed to completion. The results are presented in Tables 18-21. The arabinofuranosidic linkage was very easily cleaved, as could, of course, be expected. Since no neutral oligosaccharides were formed in any of these hydrolytic conditions, no chain of neutral sugars should be present. This was also suggested by another experiment. Oligomers of L-arabinose could be isolated by Andrews and Jones (94), and also by Stephen (95) by mild hydrolysis of polysaccharides. Under similar conditions, in the

TABLE 18

Partial hydrolysis of the water-soluble pectic acid with 0.1N oxalic acid

Component	Time of hydrolysis, hours									
	1/2	1	1 1/2	2	2 1/2	3	3 1/2	4	5	6
Acidic oligosaccharide	-	trace	+	+	+	+	+	+	+	+
Neutral oligosaccharide	-	-	-	-	-	-	-	-	-	-
Galacturonic acid	-	-	+	+	+	+	++	++	++	++
Galactose	trace	+	+	++	++	++	++	++	++	+++
Arabinose	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Rhamnose	-	-	-	-	-	-	trace	trace	trace	trace

TABLE 19

Complete hydrolysis of the degraded pectic acids (after partial hydrolysis with 0.1N oxalic acid for different periods)

Component	Time of hydrolysis, hours									
	1/2	1	1 1/2	2	2 1/2	3	3 1/2	4	5	6
Galacturonic acid	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Galactose	++	+	+	+	+	+	+	+	+	trace
Arabinose	-	-	-	-	-	-	-	-	-	-
Rhamnose	+	+	+	+	+		+	trace	trace	trace

TABLE 20

Partial hydrolysis of the water-soluble pectic acid with 45% formic acid

Component	Time of hydrolysis, hours						
	1/2	1 1/2	2 1/2	3 1/2	4 1/2	5 1/2	6 1/2
Acidic oligosaccharide	-	-	-	trace	+	+	+
Neutral oligosaccharide	-	-	-	-	-	-	-
Galacturonic acid	-	-	trace	+	++	++	++
Galactose	trace	+	++	+++	+++	+++	+++
Arabinose	+++	+++	+++	+++	+++	+++	+++
Rhamnose	-	-	-	-	-	trace	trace

TABLE 21

Partial hydrolysis of the water-soluble pectic acid with 1N sulfuric acid

Component	Time of hydrolysis, hours								
	1/2	1	1 1/2	2	2 1/2	3	3 1/2	4	5
Acidic oligosaccharide	-	+	+	+	+	++	++	++	+
Neutral oligosaccharide	-	-	-	-	-	-	-	-	-
Galacturonic acid	+	++	++	++	++	+++	+++	+++	+++
Galactose	++	++	++	+++	+++	+++	+++	+++	+++
Arabinose	+++	+++	+++	+++	+++	+++	+++	+++	+++
Rhamnose	-	-	-	-	-	-	-	trace	trace

present case, no neutral oligosaccharide could be detected on the paper chromatogram after autohydrolysis or hydrolysis with 0.1N sulfuric acid. Rhamnose was not so easily removed. The best yield of acidic oligosaccharides was realized by using N-sulfuric acid.

In another experiment a small quantity of the water-soluble pectic acid was partially hydrolyzed with N-sulfuric acid for 3 1/2 hours and the acidic oligosaccharides were separated from the neutral sugars on ion exchange resin. The acid fraction, on complete hydrolysis gave galacturonic acid (major) and rhamnose (minor) which indicated the presence of one or more acidic oligosaccharides containing these sugar residues. By the combination of ion exchange and paper chromatography it was possible to isolate four acidic oligosaccharides which had identical mobility with those obtained from the unfractionated ammonium pectate (purified).

In order to make available more of these compounds a large quantity of the unfractionated ammonium pectate (purified) was partially hydrolyzed with N-sulfuric acid. Four acidic oligosaccharides could be isolated from the hydrolyzate. Their general characteristics are shown in Table 22. The oligosaccharides containing galacturonic acid and rhamnose, which must have originated from the water-soluble pectic acid were completely characterized as described later.

Two oligomers of D-galacturonic acid which were tentatively identified as di- and trigalacturonic acids were obtained in five different ways, namely by the enzymic hydrolysis of the unfractionated ammonium pectate, by enzymic hydrolysis of the polygalacturonic acid, by enzymic hydrolysis of the water-soluble pectic acid, by partial acid hydrolysis of unfractionated ammonium pectate, and by partial acid hydrolysis of

TABLE 22

Oligosaccharides obtained by partial hydrolysis of the purified ammonium pectate (unfractionated)

Oligomer	Constituent sugar residues	$[\alpha]_D$ in water, degrees	R_{GalA} in solvent D	M_G
(a)	Galacturonic acid	+187	0.05	0.97
(b)	Galacturonic acid and rhamnose (1:2 approx.)	+118	0.10	0.73
(c)	Galacturonic acid	+154	0.20	0.95
(d)	Galacturonic acid and rhamnose (1:1 approx.)	+108	0.80	0.67

the water-soluble pectic acid. All dimers and trimers obtained by any of these methods had identical mobilities in different solvent systems and the same specific rotations. For complete characterization of these two products a larger quantity was isolated by enzymic hydrolysis of the unfractionated ammonium pectate.

Characterization of the Oligosaccharides

Oligosaccharide (d) containing galacturonic acid and rhamnose in equimolecular proportions was identified as 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose from the following observations. Reduction of the derived methyl ester-methyl glycoside with sodium borohydride followed by hydrolysis gave galactose and rhamnose. A negative color test with triphenyltetrazolium chloride in alkaline medium (82,84) indicated the presence of 2-O-substitution. The approximate molar ratio (1:1), the neutralization equivalent (345), and the R_{GalA} value (0.8) suggested that the compound was a disaccharide. After reduction with sodium borohydride and subsequent hydrolysis, no rhamnose could be detected on the paper chromatogram. The reducing end of the disaccharide accordingly consisted of rhamnose. Methylation with dimethyl sulfate and sodium hydroxide furnished a crystalline product with the same melting point as that reported by Aspinall (32) for methyl 2-O-(2,3,4-tri-O-methyl-D-galactopyranosyluronic acid)-3,4-di-O-methyl-L-rhamnopyranoside dihydrate. The methylated product, when reduced with diborane and hydrolyzed, gave 2,3,4-tri-O-methyl-D-galactose and 3,4-di-O-methyl-L-rhamnose. The α-configuration of the glycosidic linkage was assigned on the basis of the strongly positive optical rotation (Table 22). This oligosaccharide has apparently been

isolated in many cases (32,83-89), although a complete structural investigation has been carried out only in a few cases.

Oligosaccharide (b) containing approximately two parts of rhamnose and one part of galacturonic acid was shown to be O-α-D-galacturonosyl-(1 --> 2)-O-L-rhamnopyranosyl-(1 --> 2)-L-rhamnose on the basis of the following observations. The approximate ratio of the component sugars and the neutralization equivalent (505) indicated that the compound was a trisaccharide. On partial hydrolysis it gave 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnopyranose. The same disaccharide was also obtained on partial hydrolysis of the trisaccharide after prior reduction with sodium borohydride. Methylation with dimethyl sulfate and sodium hydroxide followed by methyl iodide and silver oxide furnished a product which on hydrolysis gave one spot having the R_G value of 3,4-di-O-methyl-L-rhamnose and another spot which did not move in solvent H and which was probably 2,3,4-tri-O-methyl-D-galacturonic acid. The trisaccharide did not give any color with triphenyltetrazolium hydroxide.

Oligosaccharide (c) consisting of only galacturonic acid was identified as 4-O-(α-D-galactopyranosyluronic acid)-D-galacturonic acid from the following observations. The compound had $[\alpha]_D$ +154.3 in water in good agreement with the reported value (98). It gave a positive color test with triphenyltetrazolium hydroxide. Using the procedure of Jones and Reid (99) the compound was converted to a galactobiose which was chromatographically and ionophoretically indistinguishable from an authentic sample of 4-O-α-D-galactopyranosyl-D-galactopyranose.

Oligosaccharide (a) also gave only galacturonic acid on hydrolysis and was identified as O-α-D-galactouronosyl-(1 --> 4)-O-α-

D-galacturonosyl-(1 --> 4)-α-D-galacturonic acid on the basis of the following observations. The compound had $[\alpha]_D$ +187.2, in water in an excellent agreement with the reported value (98) for the trigalacturonic acid. It gave positive color test with triphenyltetrazolium hydroxide. On partial hydrolysis it gave oligosaccharide (c), galacturonic acid and trace of the original trisaccharide. The latter was converted to its methyl ester-methyl glycoside, reduced with sodium borohydride, methylated with methyl sulfate and sodium hydroxide followed by methyl iodide and silver oxide, reduced again with lithium aluminum hydride and finally methylated with methyl iodide and silver oxide to furnish a product which was hydrolyzed with N-sulfuric acid to give 2,3,4,6-tetra-O-methyl-D-galactose, 2,3,6-tri-O-methyl-D-galactose and a di-O-methyl-D-galactose in an approximate ratio of 0.8:1.6:1. The presence of the di-O-methyl-D-galactose was evidently due to incomplete methylation. Its electrophoretic mobility indicated a 2,3-substituted sugar.

Infrared Spectra of Oligomers and Polymers

The infrared spectrum is often very useful in the structural investigation of oligo- and polysaccharides. Glucose-containing oligosaccharides exhibit absorption bands at 840 $\pm$ 8 cm^{-1} and 894 $\pm$ 7 cm^{-1} which are said to be characteristic of α- and β-links, respectively (51). Recently, Reintjes and his co-workers (109) reported some infrared spectra of different pectins.

The infrared spectra of the unfractionated ammonium pectate, the water-insoluble polygalacturonic acid and the water-soluble pectic acid (Fig. 12), and those of the oligosaccharides (Table 22) as shown

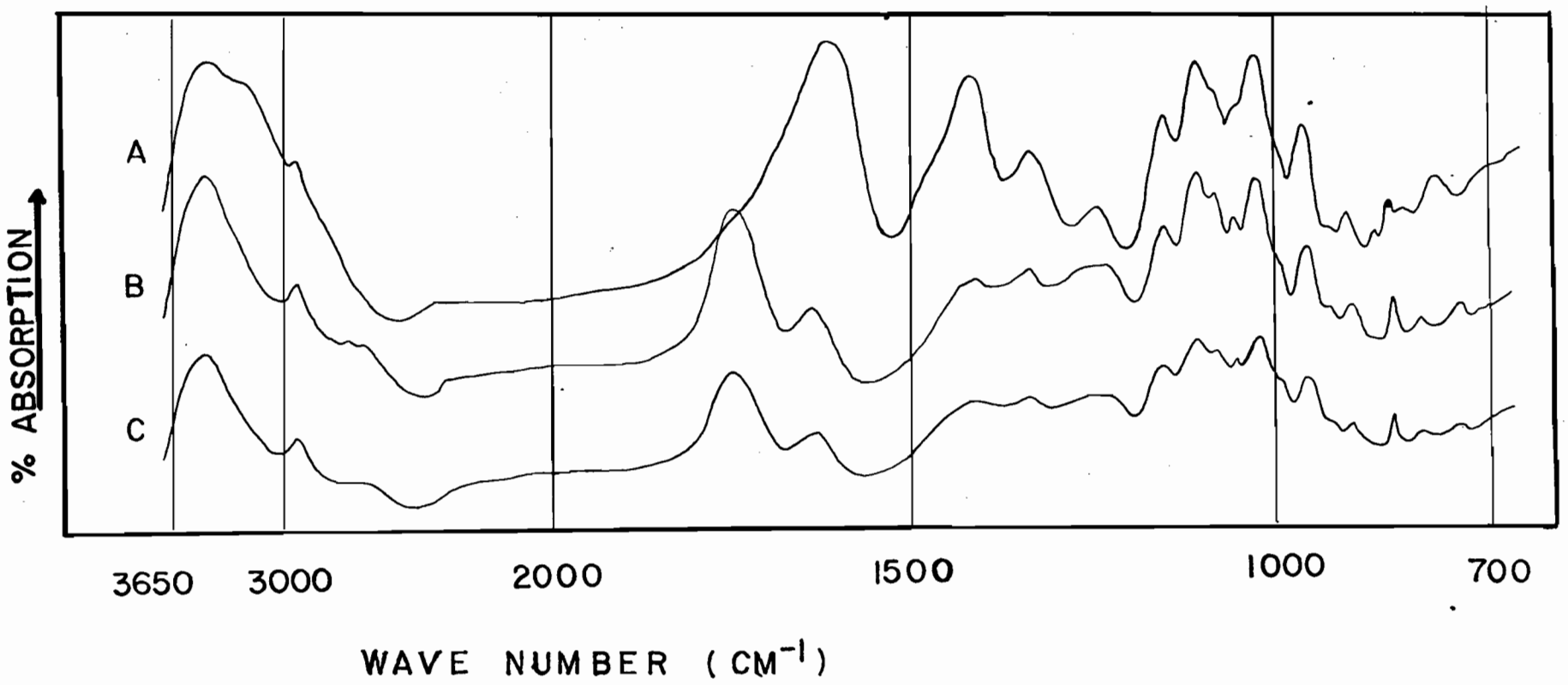
FIGURE 12

Infrared spectra of

A. Purified ammonium pectate

B. Polygalacturonic acid

C. Water-soluble pectic acid


A
B
C
% ABSORPTION
3650
3000
2000
1500
1000
700
WAVE NUMBER (CM^{-1})

in Fig. 13 were recorded. It is evident that the water-insoluble and water-soluble pectic acids had almost identical spectra. All the oligomers also had similar spectra. In all cases absorption bands were present at 3420 ± 20 and 3400 ± 20 cm^{-1}, respectively, which could be attributed to hydrogen bonded OH-stretching vibrations. The bands at 1750 cm^{-1} for the pectic acids and at 1740 ± 5 cm^{-1} for the oligosaccharides were due to C=O stretching vibrations of either ester or lactone groups. Since there were no ester groups in the oligosaccharides and the polygalacturonic acid and only negligible amounts in the water-soluble pectic acid, this band was probably due to the presence of a lactone, indicating that lactonization of the galacturonic acid moiety easily occurred on concentration in vacuo at an elevated temperature or, more likely, during the process of drying the solid or syrup over calcium chloride in vacuo. When the substances were redissolved in ordinary distilled water, an acidic pH was clearly observed indicating that if a lactone ring (presumably between C-6 and C-3) was present at all, it was easily opened by water. D-galacturonic acid after a similar treatment of concentration and drying showed a shift of C=O stretching frequency from 1716 cm^{-1} to 1760 cm^{-1} and the material showing absorption at 1760 cm^{-1}, when redissolved in ordinary distilled water showed acidic pH. This observation also, perhaps, indicates the formation of unstable lactone ring in D-galacturonic acid (101).

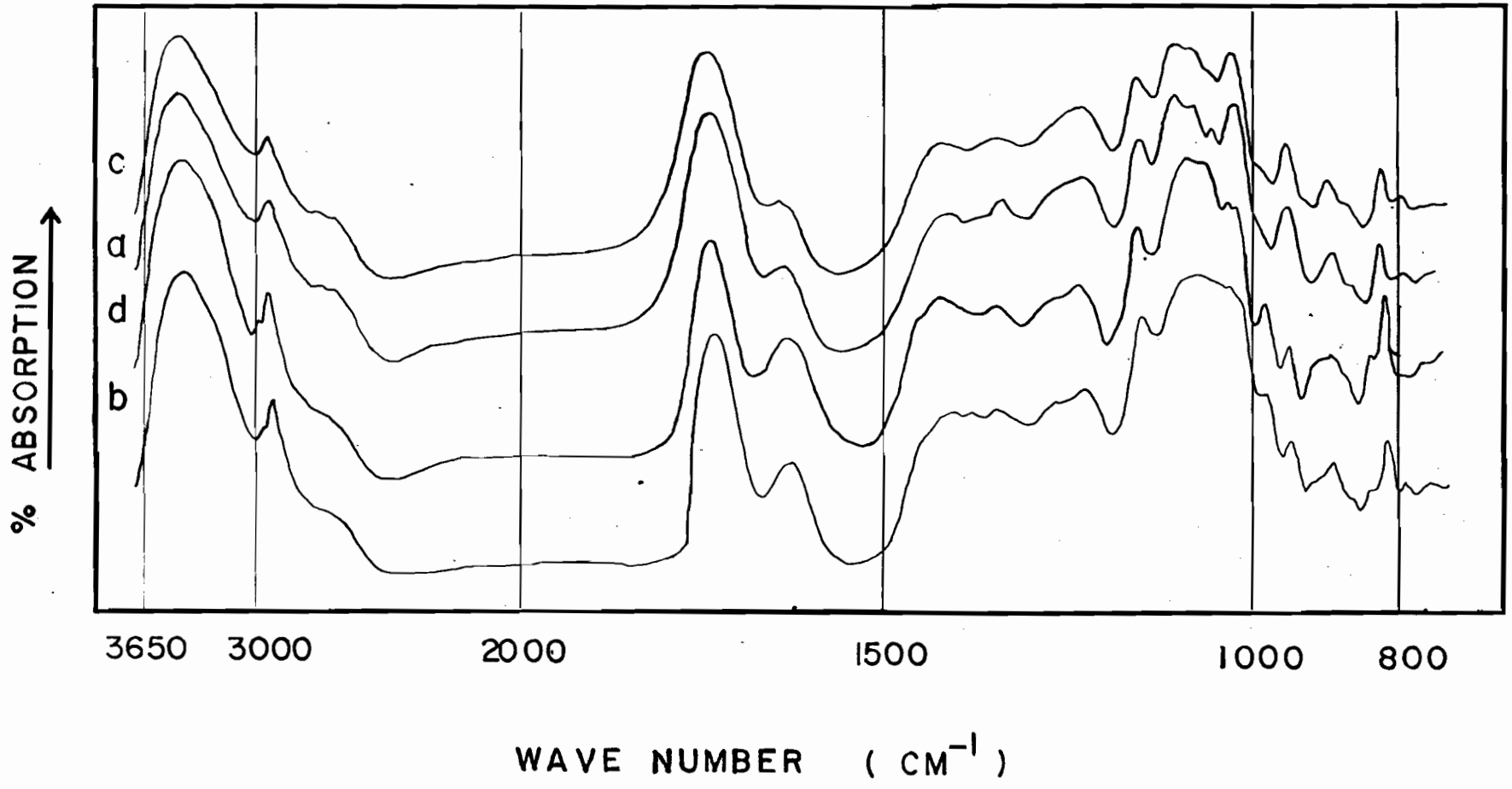
Possible Structural Features

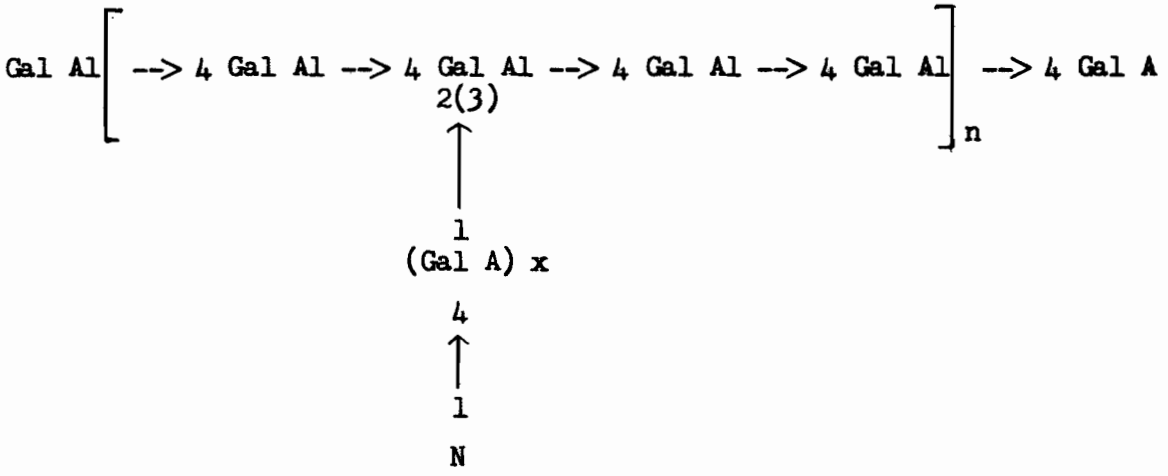
Based on the methylation data, two possible structures can be drawn for the water-soluble pectic acid, namely (I) and (II) as shown below (where rhamnose has been omitted). Since no oligomer of galact-

FIGURE 13

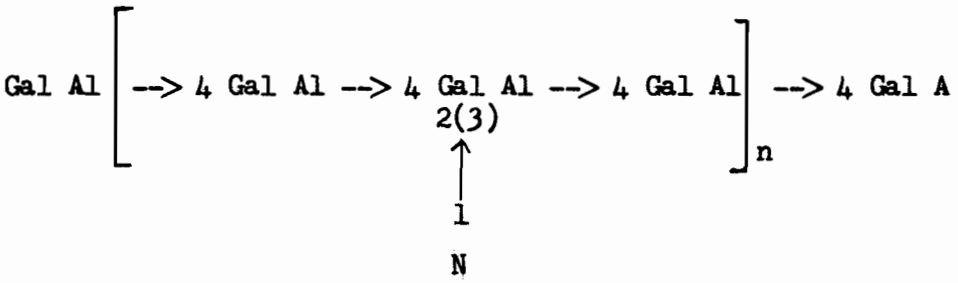
Infrared spectra of oligosaccharides

(a, b, c and d as in Table 22)


c
a
d
b
% ABSORPTION
3650
3000
2000
1500
1000
800
WAVE NUMBER (CM^{-1})



Structure I



Structure II

Gal A = α-D-galactopyranosyluronic acid

N = D-galactopyranose or

L-arabinofuranose.

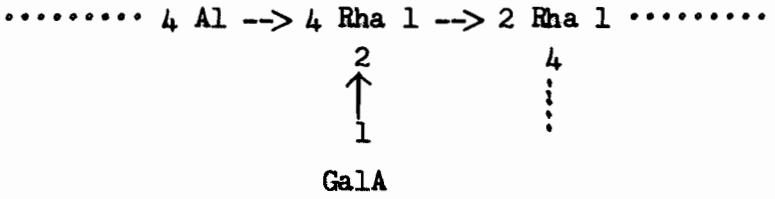
uronic acid having (1 --> 3)- or (1 --> 2)-linkage could be isolated structure I is less likely.

Both the methylation and partial hydrolysis results suggested that rhamnose must be part of the galacturonic acid framework. The isolation of the oligosaccharides (III) and (IV), and the fact that only

(III) (IV)

Rha = L-rhamnopyranose

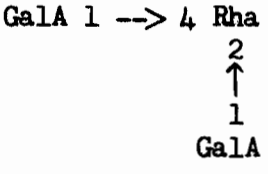
3-O-methyl-L-rhamnose was obtained from the methylated polymer are consistent with a structure of the type (V) below, where A could be



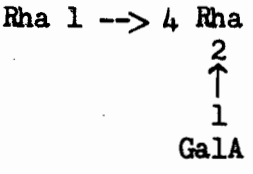
Structure V

either galacturonic acid or rhamnose, the latter is less likely in view of the formation of only 3-O-methyl rhamnose.

If A carries no substitution at C-2, it is possible that a (Al --> 4 Rha)-bond would be more susceptible to acid hydrolysis than the (Rha 1 --> 2 Rha)-bond (74). Assuming that glycosidic hydrolysis proceeds through the cyclic mechanism (90,91) and that the formation of the carbonium ion requires a transformation from the puckered chair form to a planar half-chair form (92), the large bulky substituent (galacturonic acid) on C-2 of the rhamnose moiety will diminish its tendency to assume the half-chair conformation. This resistance could be sufficient to cause a lower rate of hydrolysis for the glycosidic bond (Rha 1 --> 2 Rha) relative to (Al --> 4 Rha) in structure V, and would explain the formation of (IV) rather than (VI) or (VII). From

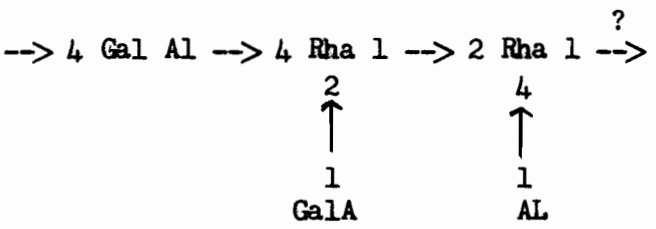


(VI)



(VII)

the present investigation it was not certain whether some rhamnose residues existed as reducing end groups, substituted at C-4. The absence of any 3,4-di-O-methyl-L-rhamnose suggested that such units must exist. Isolation of compound (IV) would indicate the attachment of an acid-labile residue. Therefore, a modified form of structure V could be present such as that shown below (VIII).



AL = sugar residue such as arabinofuranose, containing an acid-labile glycosidic linkage.

Structure VIII

CONCLUSIONS

Pectin obtained from the bark of amabilis fir consisted of two fractions, namely a water-insoluble polygalacturonic acid (galacturonan) and a water-soluble pectic acid, containing mostly galacturonic acid, but also galactose, arabinose and rhamnose residues. This is the first time such a resolution of a pectic material has been achieved. The polygalacturonic acid was composed of about 450 (1 --> 4)-linked α-D-galactopyranosyluronic acid residues (Fig. 14). The water-soluble pectic acid consisted of a linear backbone of (1 --> 4)-linked α-D-galactopyranosyluronic acid residues. L-Rhamnopyranose residues were present at the reducing end or within the main chain. The majority of the D-galactopyranose and L-arabinofuranose occurred as non-reducing end groups, probably attached as single unit side chains to C-2 or C-3 of the D-galacturonic acid residues in the backbone (Fig. 15).

The water-insoluble polygalacturonic acid was homogeneous. Although the unequivocal proof for the homogeneity of the water-soluble pectic acid was missing, the neutral sugars must be integral parts of the pectic acid.

It appears that the water-soluble pectic acid has many identical structural features with the lucerne pectic acid investigated by Aspinall and Fanshawe (32). In both cases, the absence of 2,3,6-tri-O-methyl-D-galactose in the hydrolyzates of the methylated polysaccharides definitely shows that a (1 --> 4)-linked pectic galactan is not always associated with pectic acid. On the other hand, most of the galactose residues appeared as 2,3,4,6-tetra-O-methyl-D-galactose, indicating that they exist as terminal units. An indication of the

FIGURE 14

Structural formula for galacturonan

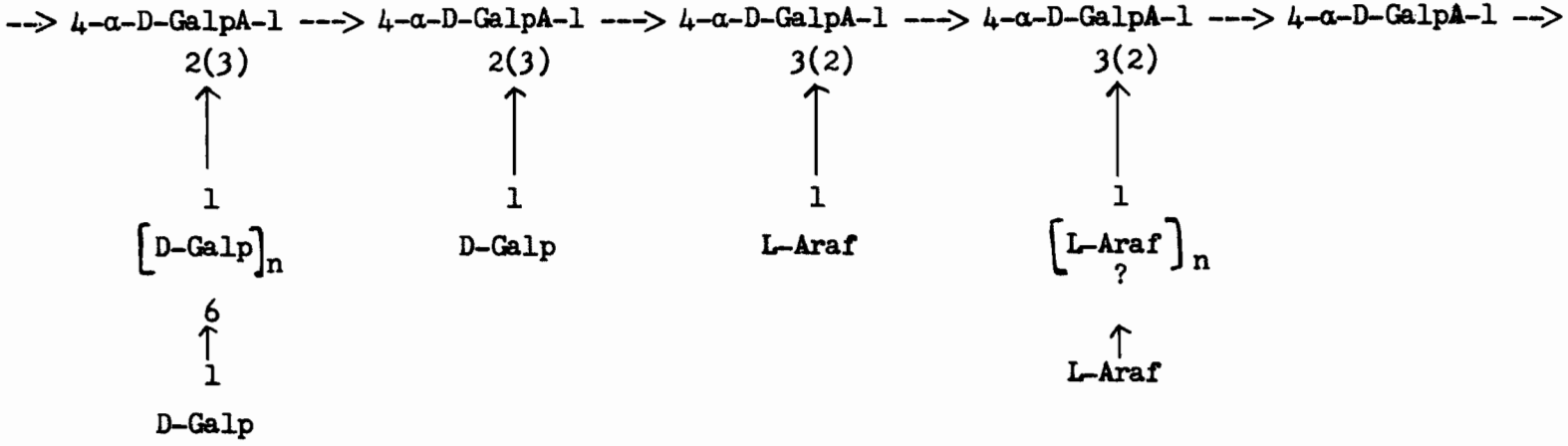
--> 4-α-D-GalpA-1---> 4-α-D-GalpA-1---> 4-α-D-GalpA-1--->4-α-D-GalpA-1-->

Galacturonan

(Polygalacturonic acid)

FIGURE 15

Tentative structure for water-soluble pectic acid


--> 4-α-D-GalpA-1 ---> 4-α-D-GalpA-1 ---> 4-α-D-GalpA-1 ---> 4-α-D-GalpA-1 ---> 4-α-D-GalpA-1 -->
2(3)
2(3)
3(2)
3(2)
1
1
1
1
[D-Galp]n
D-Galp
L-Araf
[L-Araf]n
?
6
1
L-Araf
D-Galp

Pectic Acid

presence of few (1 --> 6)-linked galactose residues was obtained in both cases, although no O-(galactosyluronic acid) galactose as obtained from lucerne pectic acid could be isolated in the present case. A similar oligosaccharide, however, was probably present in the partial hydrolyzate of the crude fir pectin. It is possible that the polysaccharide giving rise to the formation of such an oligosaccharide was removed during purification of the crude pectin. Isolation of 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose and a trisaccharide containing galacturonic acid (1 part) and rhamnose (2 parts) from both pectic acids suggests that rhamnose is an integral part of pectic acid. In the present case more information could be obtained about this trisaccharide which shows that the rhamnose residues are probably joined together through a β-L-(1 --> 2)-linkage. Unlike lucerne pectic acid, no 3,4-di-O-methylrhamnose could be obtained in the present case. The state of the arabinose is almost the same in both cases, existing as terminal units in the furanose form. Isolation of a di-O-arabinose, which was not completely identified in the present case leaves some possibility for their existence as inner units. A small quantity of this could, however, arise due to incomplete methylation or demethylation during hydrolysis. It could be possible that some 2,3-di-O-methyl-L-arabinose was obtained because of formation of L-arabinose residues by decarboxylation of D-galacturonic acid during extraction, but in the light of the present investigation this probability is less likely. Finally, it is important to note that unlike fir pectin lucerne pectin might not contain any high molecular, water-insoluble polygalacturonic acid.

It appears that "pectic acid" of the so-called pectic triad is a physical mixture of acidic polysaccharides, some based entirely on D-

galacturonic acid, and some consisting of D-galacturonic acid, D-galactose, L-arabinose and, perhaps, L-rhamnose in proportions which might vary to a considerable extent depending on the origin of the pectin. It is quite likely that most of the neutral sugar residues occur in side chains. It is also possible that pectic substances, like hemicelluloses, contain a family of closely related molecular species.

EXPERIMENTAL

All evaporations were carried out under reduced pressure below 45°C. Optical rotations were measured at 546 mμ and 578 mμ at 23° ± 3°C using a Zeiss photoelectric polarimeter; the specific rotations were equilibrium values referred to 589 mμ (sodium-D-line) with the aid of the Drude equation. Unless otherwise mentioned, rotations were observed with water solutions. All melting points were corrected. The methoxyl determinations were carried out according to a standard procedure (117, 118). Demethylations of the methylated sugars were performed with boron trichloride (104) and the products were examined by paper chromatography. Unless otherwise specified, infrared spectra were obtained with a Unicam S.P. 100 double beam prism grating spectrophotometer. The potassium bromide pellet technique was used.

Paper Chromatography

Chromatographic separations were carried out at room temperature by the descending technique on Whatman No. 1 filter paper. For preparative purposes, Whatman No. 3 MM paper stitched with a wick of No. 50 was used. R_{GalA} values of the acidic oligosaccharides refer to rate of movement relative to D-galacturonic acid and R_G values of the methylated sugars were taken as the movement relative to 2,3,4,6-tetra-O-methyl-D-glucose. The following solvent systems (v/v) were used for paper chromatography:

(A) ethyl acetate:acetic acid: water (9:2:2)

(B) ethyl acetate:acetic acid: water (9:3.5:2)

(C) ethyl acetate:acetic acid:water (10:5:6)

(D) ethyl acetate:acetic acid:formic acid:water (18:3:1:4)

(E) ethyl acetate:pyridine:water (8:2:1)

(F) n-butanol:pyridine:water (10:3:3)

(G) n-butanol:ethanol:water (4:1:5, upper layer)

(H) 2-butanone:water:ammonia (90:8:2); the mixture was saturated for 24 hours at 4°C and the non-aqueous layer was used.

(I) n-butanol:ethanol:water (5:1:4, upper layer).

Paper Electrophoresis

Paper electrophoresis was carried out in 0.05M borate solution at 700 volts using Whatman No. 3 MM paper. All M_G values, defined as the relative movement of any sugar with respect to D-glucose, were corrected for movement due to electroendosmosis by reference to 2,3,4,6-tetra-O-methyl-D-glucose or 2,3,4,6-tetra-O-methyl-D-galactose.

Sugars were located on the chromatograms and electrophoretograms by spraying with a solution of o-aminodiphenyl in glacial acetic acid (105).

Free Boundary Electrophoresis

Tiselius electrophoresis was carried out with a Spinco Model H apparatus. Photographs were taken at the initial, intermediate and final stages. The polysaccharide concentration was 2%. Sodium chloride (0.1N) and sodium tetraborate (0.05M) solutions were used.

Hydrolysis of the Polysaccharides

Hydrolysis of all polysaccharides was carried out by the method of Saeman and co-workers (106). The polysaccharide was treated with 72% sulfuric acid to form a paste and heated at 30°C for about 45 minutes. The contents were transferred to a small beaker, diluted with water (28

times the volume of sulfuric acid) and the solution was autoclaved for 75 minutes at a steam pressure of 15 p.s.i. After neutralization with barium carbonate and subsequent treatment of the filtrate with Amberlite IR-120(H) exchange resin, the solution was concentrated and stored in the cold.

Quantitative Sugar Estimation

The hydrolyzates of the polysaccharides were resolved by paper chromatography in solvent E and in some cases also in solvent A. After elution with appropriate amounts of water, the various reducing sugars were estimated by the spectrophotometric method of Timell, Glaudemans and Currie (105).

PART I

Preliminary Studies

Preparation of Extractive-free Bark

The sawdust (20-80 mesh) of the bark of amabilis fir (Abies amabilis) was exhaustively extracted in a big Soxhlet apparatus with ethanol:benzene (1:2, v/v) for 24 hours and then dried in the air.

Preparation of Holocellulose

The holocellulose was prepared by the chlorite method of Wise and co-workers (107). Extractive-free bark (1200 g., on dry basis) was suspended in water (18 liters) at 75°C. Glacial acetic acid (120 ml.) was added, followed by sodium chlorite (360 g.) which was added portion-wise over a period of one hour with vigorous stirring and at 75°C. Fresh reagents (acetic acid and sodium chlorite) were added every hour. The reaction was discontinued after four hours. The contents of the flask were allowed to settle and the liquid from the top was decanted after which the oxidation was continued in the same way for another three hours. The white holocellulose was washed, first by decantation, and then on a Büchner funnel with distilled water and later with ethanol. The material was finally dried in the air. Yield: 586 g., 48.8% of the dry extractive-free bark. Yield of 49 ± 3% was generally obtained in different experiments.

Isolation of Crude Pectin (preliminary studies)

The holocellulose (50 g.) was suspended in water (1 liter) in a beaker. Potassium acetate (3.3 g.) was added and the mixture was

stirred vigorously at 75-80°C for three hours. The residue (A) obtained after removal of the water extract was thoroughly washed with water by filtration. In a similar way the residues B, C and D were obtained by extracting, in each case, 50 g. of holocellulose with water for 6, 9 and 12 hours respectively (Fig. 1). Each of these residues was extracted with 0.5% ammonium oxalate solution (1 liter) at 75-80°C for 12 hours, aliquots being taken after 2, 6, 9 and 12 hours. In this way 16 fractions were obtained, all precipitated with ethanol. The precipitates were recovered by filtration and washed with 75% ethanol, ethanol and finally with petroleum ether.

Purification of the Crude Fractions

The crude extracts were precipitated as calcium pectates by adding 10% aqueous calcium chloride. The insoluble calcium pectates were washed thoroughly with water, heated with 0.5% ammonium oxalate for 30 minutes, and then centrifuged to remove calcium oxalate. The regenerated ammonium pectates were isolated by precipitating with ethanol. Samples PA1 to PD4 (Fig. 1) were thus obtained.

Large-scale Extraction of Holocellulose with Water

The holocellulose (1200 g.) was stirred in water (18 liters) at 75-80°C for 12 hours with potassium acetate (60 g.). The solid residue was recovered by filtration and washed with hot water (20 liters). The combined filtrate and washings were concentrated in vacuo at 40-50°C to three liters, and the aqueous solution was added with stirring to ethanol (15 liters). The precipitate formed was collected by filtration, washed successively with 70% ethanol, ethanol and petroleum ether (b.p.

30-60°C) and dried *in vacuo* at room temperature over calcium chloride and potassium hydroxide to give a white powder. Yield: 42.0 g., 3.5% of holocellulose.

Isolation of Pectin from the Water-Extract

The material (40 g.) extracted from holocellulose with water was dissolved in water (1 liter) to which a 10% aqueous solution of calcium chloride (250 ml.) was added when a precipitate was formed which was isolated by centrifugation. The residue, after being washed with water, was treated with a hot aqueous solution (0.5%) of ammonium oxalate, centrifuged and the clear solution was precipitated with ethanol to give 700 mg. of ammonium pectate.

Isolation of Crude Ammonium Pectate on a Large Scale

The material remaining after extraction with water of the holocellulose was extracted with 0.5% aqueous ammonium oxalate (15 liters) for 12 hours at 75-80°C. The extract was precipitated with ethanol, filtered, washed with 70% ethanol, ethanol and then petroleum ether, and dried *in vacuo* over calcium chloride to yield a white material (130 g., 10.8% of holocellulose). Yields of 11-13% were obtained in different experiments $[\alpha]_D$ +120° ± 7°.

Purification of Crude Ammonium Pectate

The crude ammonium pectate (113 g.) was dissolved in water (2 liters) after which a 10% aqueous solution of calcium chloride (1400 ml.) was added. The resulting precipitate was washed with water (8 liters), then suspended in 4 liters of water to which 8% aqueous sodium hydroxide

(2 liters) was added, stirred well, filtered quickly, and washed with dilute acetic acid followed by water. The solid calcium pectate, thus obtained, was heated with 0.5% ammonium oxalate solution for 30 minutes at 75-80°C. The insoluble calcium oxalate was removed by centrifugation. The supernatant solution was concentrated and precipitated with ethanol. The precipitate was filtered, washed successively with 70% ethanol, ethanol and petroleum ether, and dried in vacuo over calcium chloride. Yield: 37.0 g., 32.7% of the crude product; $[\alpha]_D$ +230°; OMe, 0.9%; ash, 2.3%. Several batches of purified ammonium pectate were prepared (all with $[\alpha]_D$ +230 ± 5°) and used in subsequent experiments.

The infrared spectrum exhibited maxima at the following frequencies (cm^{-1}): 3400 (s), 3200 (s), 2925 (m), 1615 (s), 1420 (s), 1338 (m), 1244 (w), 1151 (m), 1107 (s), 1085 (s), 1052 (w), 1023 (s), 957 (m), 898 (w), 880 (w), 840 (w), 818 (w) and 775 (w).

Attempted Fractionation of the Purified Ammonium Pectate

1. Repeated precipitation with calcium chloride: The ammonium pectate was reprecipitated as calcium pectate, washed thoroughly with water and then regenerated as ammonium pectate by means of ammonium oxalate as described before. Yield: 88% of the original material.

2. Precipitation with cetyltrimethylammonium hydroxide: The precipitate was washed with water. It could not be decomposed with 5N acetic acid but instead by heating with 0.5% aqueous ammonium oxalate. The ammonium pectate was recovered in the usual way. Yield: 90% of the original material.

3. Fractional precipitation with ethanol: Ammonium pectate (1 g.) was dissolved in water (200 ml.) and ethanol was added slowly with

stirring. Three precipitates were removed by centrifugation after addition of 200 ml. of ethanol each time. Yields are given in Table 7.

4. Ion exchange chromatography on diethylaminoethylcellulose (phosphate form): The same procedure, as described below, was followed for the chromatography of the crude and the purified pectin. Diethylaminoethylcellulose (40 g.) was washed three times alternately with 0.5N hydrochloric acid and 0.5N sodium hydroxide, and finally with water. The material thus obtained was suspended in an 0.5M solution of sodium dihydrogen phosphate and brought to pH 6.0 by adding 0.5M solution of of disodium hydrogen phosphate. It was deaerated by keeping in vacuum overnight and then washed with 0.005M phosphate buffer (pH 6.0). The resulting DEAE-Cellulose (phosphate form) was put into a column and washed with water until it was free from phosphate ion.

The moderately concentrated aqueous solution of the polysaccharide (300-400 mg.) was added at the top of the column, which was eluted successively with water (400 ml.), 0.025M-(500 ml.), 0.05M-(500 ml.), 0.1M-(500 ml.), and 0.25M-(500 ml.) sodium dihydrogen phosphate (pH 6), and a gradient of sodium hydroxide (water - 0.3M NaOH; 2 liters). Fractions were collected in 15 ml. portions. The chromatographic separation was followed by examining each test tube for the presence of the polysaccharide by determining its optical rotation and also by hydrolyzing the contents (0.1 ml.) with concentrated sulfuric acid (5 ml.) in the presence of phenol (1 ml.) (108).

For the isolation of the polysaccharide from the phosphate eluate, the solution was treated with a mixture of cation and anion exchange resins, concentrated and precipitated with ethanol. To recover the polysaccharide from sodium hydroxide eluate, the latter was treated

with Amberlite IR-120(H) and then poured into ethanol. In one experiment the regenerated free acid was converted to sodium salt by means of sodium bicarbonate, precipitated again with ethanol and recovered in the usual way. Yield of the sodium pectate was 84% of the original purified ammonium pectate (Table 7). In a similar way sodium pectate could be obtained from crude pectin in a yield of 28% (Table 6).

5. Electrophoresis-convection method: The electrophoresis-convection apparatus used has been described by Yean and Goring (55). The general set-up is shown in Fig. 16. The solution was held between two co-axial nylon bags, 3.2 cm. and 5.1 cm. in diameter and about 43.2 cm. long. The inner bag was filled with distilled water and contained the platinum cathode which was prevented from touching the inner bag by means of a glass frame. The anode consisted of four 0.6 cm. x 45.7 cm. strips of platinum, mounted on a Lucite frame and placed outside the outer bag. The voltage was usually maintained between 250-400 v.

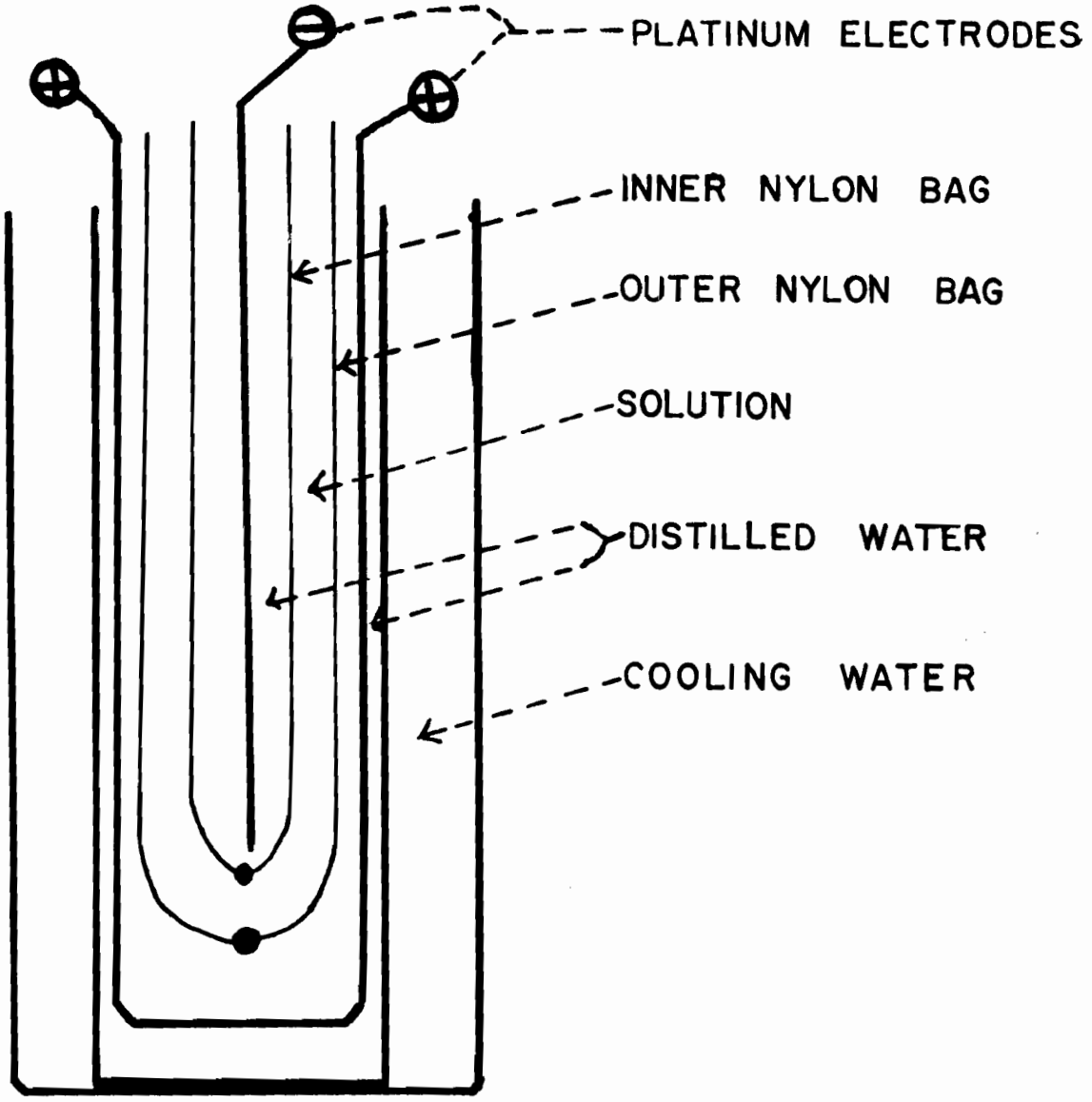
The ammonium pectate (500 mg.) was dissolved in 400 ml. of water and then placed between the inner and the outer bags. After about 5 hours a turbid viscous layer was clearly observed at the bottom of the outer tube. After 8 hours, the current was turned off and fractions were carefully removed with a syringe starting from the top layer with minimum disturbance of the contents. In one experiment with the purified ammonium pectate the turbid viscous layer was neutralized with sodium bicarbonate and the sodium pectate was recovered in a yield of 85% of the original purified ammonium pectate (Table 7).

Fractionation of the Purified Ammonium Pectate

1. Treatment with Amberlite IR-120(H): The ammonium pectate (0.5

FIGURE 16

Apparatus for electrophoresis-convection technique


PLATINUM ELECTRODES
INNER NYLON BAG
OUTER NYLON BAG
SOLUTION
DISTILLED WATER
COOLING WATER

g.), after being dissolved in water, was added to the top of a column of Amberlite IR-120(H) which was eluted with water. The turbid eluate was concentrated and subjected to ultracentrifugation at 20,000 r.p.m. (40210 g). The supernatant clear solution was treated with more Amberlite IR-120(H) until no more turbidity was observed. The resulting clear solution was concentrated and precipitated with ethanol. The precipitate was washed with ethanol and petroleum ether and then dried in vacuo over calcium chloride to give a yield of 160 mg. of the water-soluble pectic acid. The remaining solid residue was washed with water and subjected to renewal ultracentrifugation. This process was repeated in all six times. The solid material was finally washed with acetone and then with petroleum ether, after which it was dried in vacuo over calcium chloride to yield 250 mg. of the water-insoluble polygalacturonic acid (galacturonan). For large-scale fractionation a larger column of Amberlite IR-120 (H) was used. The water-insoluble polygalacturonic acid used in the subsequent experiments had a methoxyl content of 0.6%; $[\alpha]_D$ +246 $\pm$ 3° (for the sodium salt); and ash, 0.16%; and showed infrared absorption at the following frequencies (cm^{-1}): 3440 (s), 2930 (m), 2600 (w), 1750 (s), 1640 (m), 1415 (m), 1340 (m), 1235 (m), 1155 (s), 1107 (s), 1082 (s), 1055 (m), 1023 (s), 990 (m), 955 (m), 913 (w), 892 (w), 835 (w) and 795 (w). The water-soluble pectic acid used in the subsequent experiments had OCH_3, 2.7%; $[\alpha]_D$ +220° $\pm$ 5°; ash, 0.35%; the infrared spectrum showed absorption at the following frequencies (cm^{-1}): 3420 (s), 2925 (m), 2550 (w), 1750 (s), 1638 (m), 1415 (m), 1340 (m), 1235 (m), 1155 (s), 1108 (s), 1083 (s), 1055 (m), 1022 (s), 955 (m), 895 (w), 835 (w) and 795 (w).

2. Acidification with Sulfuric Acid: To the aqueous solution of purified ammonium pectate, N-sulfuric acid was added slowly with continuous

stirring until the pH was 4, when some precipitate appeared which was removed by ultracentrifugation. The solid residue of polygalacturonic acid, after being washed with water several times followed by washing with acetone and petroleum ether, was dried and recovered. The supernatant acidic solution was added to four times its volume of ethanol to produce a precipitate of pectic acid which was recovered in the usual way.

3. Electrophoresis-convection method: The turbid viscous layer obtained at the bottom of the bag, as mentioned before, was diluted with water. After being centrifuged, the supernatant solution was mixed with ethanol and the precipitate formed was recovered in the usual way. The water-insoluble portion was washed with water and then recovered as a white powder by freeze-drying.

4. Treatment with saturated sodium chloride solution: Ammonium pectate (1 g.) was dissolved in water (100 ml.) to which saturated solution of sodium chloride was added very slowly. After adding 10 ml. of the sodium chloride solution the precipitate formed therein was separated by centrifugation. The solid residue, in this case, was soluble in water. The supernatant solution was dialyzed and then precipitated with ethanol. The precipitate was recovered in the usual way.

Enzymic Hydrolysis of Unfractionated Ammonium Pectate

Ammonium pectate (1 g.) was dissolved in water (100 ml.) to which pectinase (200 mg.) was added. The solution was dialyzed in cellophane tubing (2.7 cm. diameter) against distilled water (6l) at room temperature. The dialyzate was withdrawn after 6 hours and replaced with fresh distilled water. Four fractions of dialyzate were collected

by changing water every sixth hour. The dialyzates were concentrated, treated with Amberlite IR-120(H) and fractionated by paper chromatography using solvent B. The first fraction (after 6 hrs.) contained most of the oligosaccharides.

Partial Acid Hydrolysis of Crude Pectin

The crude ammonium pectate (7.5 g.) was dissolved in water (194 ml.) and concentrated sulfuric acid (6 ml.) was added. The resulting turbid solution was heated under reflux on a boiling water bath for 3.5 hours. The degraded pectic acid was recovered by adding acetone to the cooled solution. The precipitate was recovered in the usual way. The filtrate and washings were combined and concentrated to remove acetone, neutralized with barium carbonate, filtered, and treated with Amberlite IR-120(H). The solution thus obtained contained monomers and oligomers. This partial acid hydrolyzate, after being concentrated, was chromatographed using Amberlite CG-45 (formate form). The column was eluted with water to remove all neutral sugars and then with formic acid using gradient elution (0 - 0.2N). The acidic oligosaccharides were not separated well in this case. Therefore, all fractions containing acidic oligosaccharides and some galacturonic acid were mixed and shaken with ether several times to remove formic acid. The aqueous layer was concentrated and fractionated by paper chromatography using solvent D.

PART II

The Polygalacturonic Acid

Enzymic Hydrolysis

The polygalacturonic acid was suspended in water and neutralized with sodium bicarbonate. The resulting clear solution was precipitated with ethanol and recovered in the usual way. The sodium salt of polygalacturonic acid (1.0 g.) was treated with pectinase in the same way as described before. After treatment with Amberlite IR-120(H), the hydrolyzate (0.401 g.) was resolved into five fractions, namely galacturonic acid (160 mg.), digalacturonic acid (56 mg.), trigalacturonic acid (40 mg.), tetragalacturonic acid (12 mg.) and pentagalacturonic acid (approx. 2 mg.) using paper chromatography in solvent B. The dimer, trimer, tetramer and pentamer of galacturonic acid had R_{GalA} values of 0.63, 0.39, 0.24, 0.13 in solvent B and 0.52, 0.26, 0.17 and 0 in solvent C, respectively.

Esterification and Reduction

The polygalacturonic acid (20 g.) was suspended in water (200 ml.) and propylene oxide (80 ml.) was added (25,75). The mixture was shaken slightly for one hour when the turbidity disappeared. Some heat was generated during this period. The reaction flask was cooled to room temperature and then shaken for nine days. The esterified product was recovered by precipitation with acetone. Yield of the propylene glycol ester was 21.5 g.

The glycol ester (21.5 g.) was dissolved in water (170 ml.) and an aqueous solution of sodium borohydride (21 g. in 170 ml.) was added

dropwise over a period of one hour. Some glycerol was added to maintain the pH at about 8.5 (30). The solution was allowed to stand overnight after which excess borohydride was destroyed by addition of acetic acid. The solution was passed through Amberlite IR-120(H) and concentrated to about 400 ml. Methanol (200 ml.) was added and evaporation was continued until the volume was reduced to about 200 ml., when the same volume of methanol was added and again evaporated. This treatment was repeated three times more. Finally, the viscous aqueous solution (200 ml.) was added with stirring to 2 liters of a 1:1 mixture of acetone and ethanol. The product obtained was re-esterified with propylene oxide and subsequently reduced with sodium borohydride in exactly the same way as before. After a total number of four esterification-reduction cycles, the yield was 13 g. of a white powder, having galactose and galacturonic acid in a ratio of 94:6, and this reduced product was used for methylation.

Acetylation (77)

The polygalacturonic acid (1 g.) was dispersed in water, freeze-dried and finally dried *in vacuo* over phosphorus pentoxide. It was then added to 25 ml. of formamide. The suspension was stirred for one hour at 40-50°C, during which time the polysaccharide was dispersed completely. Pyridine (75 ml.), freshly distilled over barium oxide, was added in small portions, and the mixture was cooled to 30°C. Acetic anhydride (10 ml.) was added, and the reaction mixture was shaken at room temperature for 12 hours, after which time 10 ml. of acetic anhydride was added. After a total number of three additions of acetic anhydride and a reaction time of two days, the mixture was poured into one liter of ice-cold water

containing 2% of hydrochloric acid. The precipitated ester was stirred well, isolated by filtration, washed first with 0.5% cold hydrochloric acid and then several times with ice-cold water until the washings were neutral. Water was displaced with methanol and methanol with petroleum ether. The final product was dried *in vacuo* over calcium chloride. The yield was 1.10 g.

Reduction with Diborane

The acetylated product was treated with diborane according to the general procedure of Smith and Stephen (73) which was also successfully applied by McKee and Dickey (74). The diborane was generated *in situ* over a period of 2 hours under mechanical stirring by the dropwise addition of 2 ml. of boron trifluoride etherate, diluted with 1 ml. of diglyme [bis(2-methoxyethyl)ether] to a solution of 0.4 g. of sodium borohydride dissolved in 12 ml. of diglyme. In this solution 1.1 g. of the acetylated polysaccharide was suspended. Stirring was continued for an additional 3 hours. The reaction mixture was allowed to stand overnight and was then decomposed by addition of ice-water. When evolution of hydrogen had ceased, the mixture was neutralized with 0.5N sodium hydroxide to pH 7 and poured into two volumes of ethanol. The precipitate was recovered by filtration, washed with ethanol and petroleum ether, and dried *in vacuo* over calcium chloride. Yield 0.6 g. On hydrolysis, this product gave galacturonic acid and galactose in a ratio of 3:2.

Methylation of the Reduced Polygalacturonic Acid

The reduced polygalacturonic acid (10.5 g.) was dissolved in

water (195 ml.) and sodium hydroxide (100 g.) was added with stirring in an atmosphere of nitrogen. Dimethyl sulfate (70 ml.) was added over a period of 8 hours at about 10°C. The addition of sodium hydroxide and dimethyl sulfate was repeated three times. Each time solid sodium hydroxide (85 g.) and dimethyl sulfate (170 ml.) were added. Good stirring was assured throughout by addition of water whenever necessary. After about 40 hours, the reaction product was neutralized carefully with concentrated sulfuric acid, and 50% acetic acid was added to bring the pH to 4. The solution was heated to boiling, when the methylated product separated as a cake at the surface. The latter was recovered by filtration, washed with some hot water and dried in vacuo. The combined filtrate and washings were extracted with chloroform. The chloroform extract, after evaporation, was mixed with the other portion. The combined partially methylated product was redissolved in chloroform, dried over anhydrous sodium sulfate, evaporated, and dried over phosphorus pentoxide in vacuo. The yield was 8.0 g.

The partially methylated polysaccharide (8 g.) was dissolved in dimethyl formamide (90 ml.) which had been dried over barium oxide and distilled shortly before use. Freshly prepared dry silver oxide (35 g.) and methyl iodide (35 ml.) were added to the solution and then shaken in the dark for 24 hours at room temperature. Two more similar additions of methyl iodide and silver oxide were made, and some Drierite was also added, the mixture being shaken for 24 hours each time. After a reaction time of 3 days, the mixture was filtered and the residue was washed with chloroform. Some white crystalline precipitate, presumably an addition product of silver iodide - oxide and dimethyl formamide appeared which was removed by filtration. The chloroform solution was washed with 5%

potassium cyanide (2 x 200 ml.) and water (3 x 200 ml.) and dried over anhydrous sodium sulfate. The solution was filtered through folded paper, concentrated to about 100 ml., and added slowly with vigorous stirring to 1 liter of petroleum ether. A fibrous precipitate appeared, which was washed with petroleum ether and dried over phosphorus pentoxide. Yield 3.5 g. OCH_3, calculated for a fully methylated hexosan 45.6%, found: OCH_3, 42.4%; $[\alpha]_D$ +125° (c, 1.1 in chloroform).

Methanolysis and Hydrolysis

The methylated product (2 g.) was refluxed with 0.7N anhydrous methanolic hydrogen chloride (60 ml.) for 7 hours. The resulting solution was neutralized with silver carbonate, filtered through Celite, treated with hydrogen sulfide, again filtered through Celite and evaporated to dryness. The product was dissolved in N-sulfuric acid (50 ml.) and boiled under reflux for 8 hours. The acid was neutralized with barium carbonate, filtered through Celite, washed with distilled water, concentrated to a small volume, treated with Amberlite IR-120(H), filtered, and evaporated to give a sirup. Yield 1.6 g.

Separation of Methylated Sugars

Paper chromatographic examination of the sirupy mixture of methylated sugars in solvents G and H suggested the presence of three components. A portion of the sirup was fractionated by using Whatman No. 3 MM filter paper with a wick of Whatman No. 50 paper in solvent H. The various fractions were eluted quantitatively from the chromatograms with water and concentrated to a small volume. One fraction which did not move from the starting line was treated with Amberlite IR-120(H) and Darco

G-60 charcoal. The other fractions were treated with a mixture of Amberlite IR-120(H), IR-45(OH) exchange resins and with Darco G-60 charcoal. The solids were removed by filtration (Whatman No. 5 filter paper) and washed with an alcohol:water (1:1) mixture until the washings gave a negative Molisch test. After evaporation to dryness, the solid residue was extracted with ethyl acetate, which was filtered. Evaporation to dryness gave clear pale yellow sirups which were dried in vacuo over phosphorus pentoxide.

Preparation of Methylated Galactan and Hydrolysis

The methylated polysaccharide still containing some uronic acid residues was further reduced and then methylated. A small quantity (0.8 g.) was dissolved in dry tetrahydrofuran (15 ml.) to which lithium aluminum hydride (0.5 g.) in tetrahydrofuran (25 ml.) was added slowly, after which the mixture was refluxed for 2 hours. The reduced methylated polysaccharide, after separation from inorganic salts by treating with cation and anion exchange resins, was methylated twice with methyl iodide and silver oxide to give a methylated galactan (0.4 g.), $[\alpha]_D$ +128° (c, 1.0 in chloroform). Found OCH_3, 42.7%. Methanolysis and subsequent hydrolysis were carried out as described before. Three spots could be detected on the paper chromatogram. In addition to 2,3,4,6-tetra-O-methyl-D-galactose (minor) and 2,3,6-tri-O-methyl-D-galactose (major part) another spot corresponding to 2,3-di-O-methyl-D-galactose was observed.

Identification of Methylated Sugars

Fractions a, b, and c as shown in Table 11 were identified by comparing the R_G values, and specific rotations with values reported in

the literature and finally by their conversion to crystalline derivatives.

2,3,4,6-Tetra-O-methyl-D-galactose [fraction (a)] - The sirup which had $[\alpha]_D$ +102° (c, 0.5) and R_G values 0.9 and 0.8 in solvents G and H, respectively, was characterized by conversion to its aniline derivative which was prepared by refluxing the sirup with a mixture of aniline and ethanol (110). Removal of the solvent by slow evaporation gave only a few crystals, which were carefully removed and recrystallized from ethanol to give m.p. and mixed m.p. 196-198°C.

2,3,6-Tri-O-methyl-D-galactose [fraction (b)] - This sirup had $[\alpha]_D$ +84° (c, 1.1 in water), and R_G values 0.73 and 0.5 in solvents G and H, respectively. It was converted to the corresponding aldonic acid by oxidation with bromine (111). After lactonization in vacuum at 60°C for 2 hours, the product (2,3,6-tri-O-Me-D-galactonolactone) crystallized. It was recrystallized from ethyl ether, m.p. and mixed m.p. 97-98°C.

The methylated uronic acid [fraction (c)] - This minor fraction which did not move in solvent H was treated with Amberlite IR-120(H) and then completely dried. The product was refluxed with anhydrous methanolic hydrogen chloride (0.7N). The resulting product was reduced with lithium aluminum hydride in tetrahydrofuran. After hydrolysis of the glycoside with N-sulfuric acid, the product gave only one spot in solvent G and H corresponding to a di-O-methylgalactose. The aniline derivative had m.p. 130-132°C which agreed with the reported values for aniline derivative of 2,3-di-O-methyl-D-galactose (102,103).

Determination of the Number-Average Molecular Weight of the Methylated Galactan

The osmometric measurements were carried out with modified (112) Zimm-Myerson type (113) osmometers, provided with gel cellophane membranes. The solvent was a mixture of chloroform and ethanol (9:1, v/v). The temperature was maintained at 30 ± 0.01°C and the static method of measuring the osmotic height was used. The results obtained are presented graphically in Fig. 17. From the plot the value of $(h/W)_{W=0}$ was 0.28, corresponding to a molecular weight of 91,785 and an approximate number average D.P. of 450.

Synthesis of 2,3,4,6-tetra-O-methyl-D-galactose

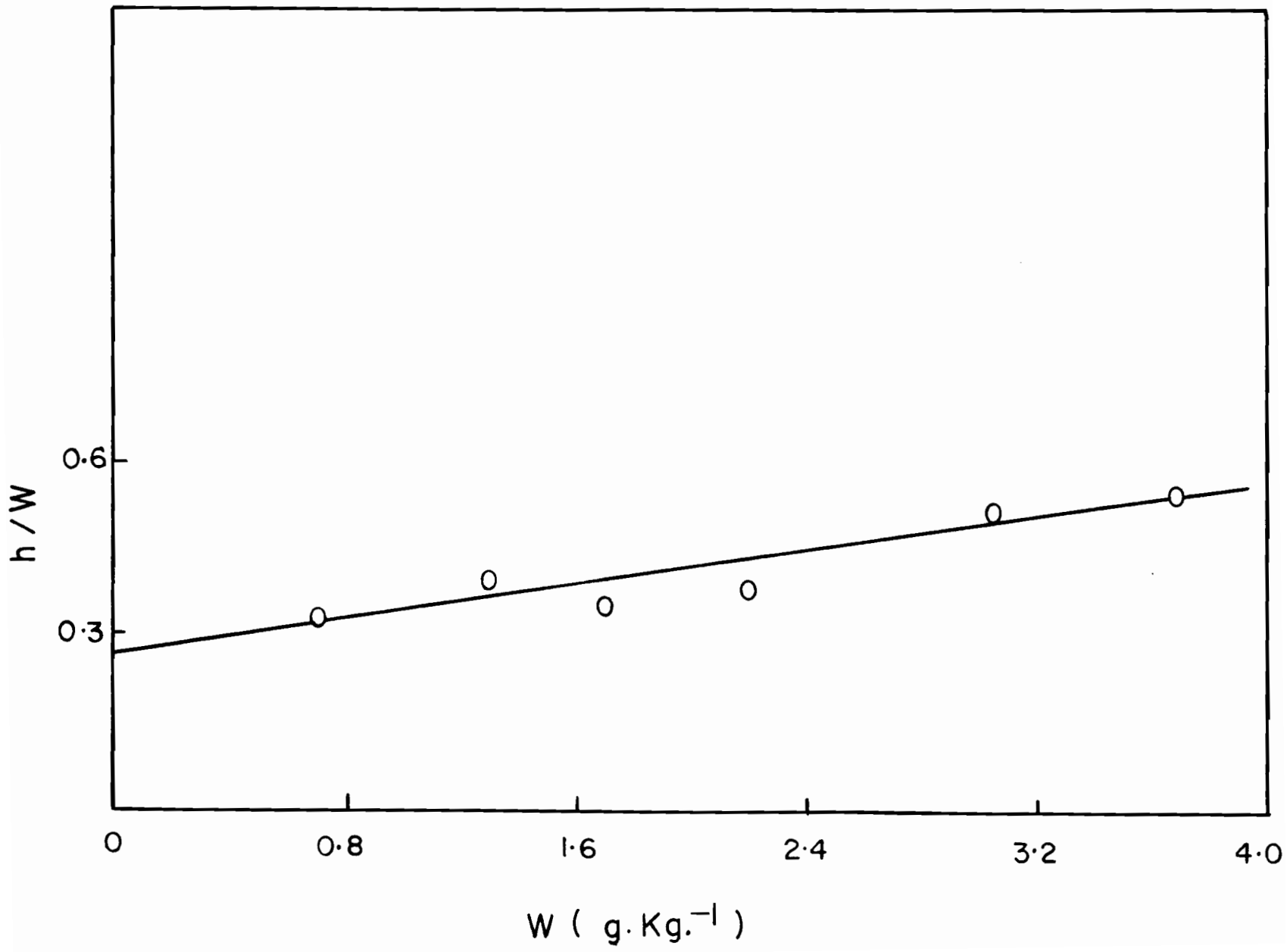
D-Galactose (100 g.) was methylated by the Haworth procedure (68), using dimethyl sulfate in excess at the initial stage. The methylated product, obtained by extracting the reaction mixture with chloroform, was purified by distillation in high vacuum to give a sirupy mixture (92.5 g.) of α- and β-galactosides. A portion of this was hydrolyzed with N-sulfuric acid to give 2,3,4,6-tetra-O-methyl-D-galactose, which was chromatographically pure and formed crystalline aniline derivative having m.p. 193-194°C.

FIGURE 17

Osmometry data for methylated galactan

h = the height of solution (cm.)

W = concentration (g./kg.)


h / W
0·6
0·3
0
0·8
1·6
2·4
3·2
4·0
W (g. Kg.⁻¹)

PART III

The Water-Soluble Pectic Acid

Attempted Fractionations

Attempts were made to fractionate the water-soluble pectic acid using calcium chloride, cetyltrimethylammonium hydroxide, ethyl alcohol and diethylaminoethylcellulose (phosphate form) in the same way as described before in the case of unfractionated ammonium pectate (Part I).

Methylation

The water-soluble pectic acid (8 g.) was dissolved in water (200 ml.) and sodium hydroxide (70 g.) was added. Dimethyl sulfate (70 ml.) was added dropwise at 10°C during six hours while the reaction mixture was being vigorously stirred, and nitrogen was passed through the system. The addition of sodium hydroxide (70 g.) and dimethyl sulfate (70 ml.) was repeated once more. After 20 hours the contents of the flask were diluted with water and neutralized with sulfuric acid, after which the pH was adjusted to 5.5 by addition of acetic acid. No precipitate was formed when the solution was heated to boiling. The solution was cooled and extracted with chloroform. From the chloroform extract only a few milligrams of material could be obtained. The solution was dialyzed against running tap water to remove all salts, concentrated and treated with silver carbonate. The silver salt was freeze-dried and then dried *in vacuo* over calcium chloride and potassium hydroxide. The dried silver salt (5.1 g.) was refluxed with a mixture of dry methanol (25 ml.) and methyl iodide (75 ml.) for 4 hours. Some Drierite was added together with silver oxide (4 g.) and the reaction was continued for another 4 hours.

The product obtained after removal of the inorganic substances was methylated three times with methyl iodide (3 x 75 ml.) and silver oxide (3 x 4 g.) by shaking the reaction mixture at room temperature for a total period of five days. The methylated product (1.5 g.) had $[\alpha]_D$ +118° (c, 0.6 in chloroform); OCH_3, 39.2%, not raised by further methylations.

Hydrolysis of the Methylated Polysaccharide and Resolution of the Hydrolyzate

N-Sulfuric acid (30 ml.) was added to the methylated methyl pectate (1.2 g.) and the solution was refluxed for 12 hours. At first, the methylated polysaccharide did not dissolve completely, but after 20-30 minutes the solution became clear. The solution was neutralized with barium carbonate, filtered through Celite, and washed with water. The combined filtrate and washings, after treatment with Amberlite IR-120(H), was concentrated to a small volume at 30°C and added to the top of a column of Dowex 1-X4 (acetate) exchange resin. The column was first eluted with water until the eluate showed a negative Molisch test. The aqueous eluate was concentrated at 30° ± 2°C to a sirup (270 mg.) which was resolved into six fractions (N1 to N6) as shown in Table 14 by means of paper chromatography in solvent H. The column was then eluted with 30% acetic acid until the eluate showed a negative test for sugar. The acid solution was concentrated several times with water to remove acetic acid completely and then dried to a sirup (645 mg.) which was a mixture of different methylated uronic acids.

Methanolysis of the Mixture of Methylated Uronic Acids and Reduction, Hydrolysis, and Separation of Methylated Sugars

The acid sugar mixture (645 mg.) was refluxed with anhydrous methanolic hydrogen chloride (1N) for 6 hours. The acid was neutralized with silver carbonate, and the solution was filtered through Celite, washed with methanol, treated with hydrogen sulfide, filtered through Celite again, concentrated to a sirup, and dried over phosphorus pentoxide. The dry sirup was dissolved in tetrahydrofuran (50 ml.) and lithium aluminum hydride (1.0 g.) was added slowly during one hour, while the mixture was being refluxed. The mixture was refluxed for another hour after which the excess hydride was destroyed by addition of ethyl acetate and water. The insoluble material was removed by centrifugation and extracted with ethanol and acetone. The combined supernatant liquid and extracts were concentrated to a sirup which was refluxed with N-sulfuric acid for 7 hours. After neutralization with barium carbonate, the mixture of neutral methylated sugars was recovered in the usual way and resolved into five fractions (A1 to A5) as shown in Table 13. Fraction A4 was further resolved into two components. In both cases separation was achieved by paper chromatography in solvents H and A, respectively.

Identification of the Methylated Sugars

Fraction A1: 2,3,4-tri-O-methyl-D-galactose

The aniline derivative of this fraction was prepared by refluxing 5 mg. of the sample with 2 drops of aniline and 1.0 ml. of ethanol. On evaporation a few crystals appeared, with m.p. and mixed m.p. 164-166°C.

Fraction A2: 2,3-di-O-methyl-D-galactose

This fraction had an M_G value of 0.32; calcd. for $C_8H_{16}O_6$: OMe, 29.8%. Found: OMe, 28.8%. R_G values are given in Table 13. An aliquot of this fraction (40 mg.) was refluxed with aniline (40 mg.) and ethanol (2.5 ml.) for 3 hours. After evaporating the solvent, a small quantity of ether was added to the product when crystals appeared. The latter were recrystallized from a mixture of ethanol:ethyl ether (1:1); m.p. 130-131°C. Different melting points have been reported in the literature for the anilide of 2,3-di-O-methyl-D-galactose as shown below. The m.p. obtained in the present case agrees well with some of them.

Melting points of the anilide of 2,3-di-O-Me-D-galactose	References
130-131°C	(103)
128-129°C	(102)
139-140°C	(32)
154-155°C	(114)

Fraction A4(i): 3-O-methyl-D-galactose

This compound gave color reaction with triphenyltetrazolium hydroxide. The M_G and R_G values are given in Table 13. It had a higher M_G value than 2-O-methyl-D-galactose. Calcd. for $C_7H_{14}O_6$: OMe, 16.0%. Found: OMe, 15.13%.

Fraction A4(ii): 2-O-methyl-D-galactose

This fraction gave no color reaction with triphenyltetrazolium chloride in alkaline medium. The M_G and R_G values are presented in Table 13. On long standing at room temperature the sirup crystallized, m.p. 145.5°C. Calcd. for $C_7H_{14}O_6$: OMe, 16.0%. Found: OMe, 14.9%. The aniline derivative of this fraction crystallized; m.p. 159-161°C (115).

Fraction A5: D-galactose

This product was chromatographically and ionophoretically indistinguishable from D-galactose.

Fraction N1: 2,3,5-tri-O-methyl-L-arabinose

On demethylation this fraction gave only arabinose. The methylated sugar (20 mg.) was dissolved in water (5 ml.) to which seven drops of bromine were added and kept in the dark for 48 hours. Excess bromine was removed by aeration and the solution was neutralized with silver carbonate. The filtrate was treated with Amberlite IR-120(H) and concentrated at 90°C in vacuo for about 30 minutes to yield the lactone. Treatment of the lactone with anhydrous methanolic ammonia at room temperature for 24 hours furnished a crystalline product, 2,3,5-tri-O-methyl-L-arabonamide, m.p. and mixed m.p. 134-135°C.

Fraction N2: 2,3,4,6-tetra-O-methyl-D-galactose

This compound was chromatographically and ionophoretically pure and was characterized as 2,3,4,6-tetra-O-methyl-D-galactose by its conversion to the aniline derivative, which had m.p. and mixed m.p. 193-195°C.

Fraction N3: di-O-methyl-L-arabinose

On demethylation, this fraction gave mainly arabinose and a trace of xylose. The aniline derivative of this component could not be crystallized. The R_G values (Table 14) suggested that it might be a di-O-methyl-L-arabinose.

Fraction N5: 3-O-methyl-L-rhamnose

On demethylation this compound gave only rhamnose. The M_G and R_G values are given in Table 14. It had $R_{rhamnose}$ 1.48 and 1.42 compared to the values of 1.50 and 1.45 in solvents D and F reported (116) for

3-O-methyl-L-rhamnose. $R_{rhamnose}$ in solvent G was 1.5. Fractions N1, N2 and N5 were examined with thin layer (Silica Gel G) chromatography using solvent H with sulfuric acid as the spraying reagent. All sugars gave single spot in different locations. After long standing at room temperature, few crystals appeared in the sirup of Fraction N5, some of which were removed, washed with methanol and then dried on a watch-glass in vacuo over calcium chloride. M.p. 106-108°C. Reported m.p. for 3-O-methyl-L-rhamnose 111-114°C (32).

Enzymic Hydrolysis

Enzymic hydrolysis of the water-soluble pectic acid (1 g.) with pectinase (200 mg.) was carried out in the same way as described in Part I. For the comparative study with the polygalacturonic acid (Na-salt), the sodium salt of the water-soluble pectic acid was prepared and subjected to the action of pectinase.

Inhibited Enzymic Hydrolysis

In this case the purified ammonium pectate (unfractionated) was used. Ammonium pectate (1.0 g. dissolved in 50 ml. of water), pectinase (200 mg. dissolved in 50 ml. of water) and arabonolactone (4 g. dissolved in 25 ml. of water) were mixed together and kept at room temperature for five hours. The solution was then heated on a water-bath to inactivate the enzyme. The cooled solution was dialyzed against distilled water for 12 hours. The dialyzate was concentrated and kept at 4°C for several days when a considerable quantity of arabonolactone separated out. The clear solution from the top was decanted and treated with Amberlite IR-120(H). The crude hydrolyzate was passed through a column of Dowex 1-X4

(acetate), which was washed with water until the eluate gave a negative test for sugar, after which the column was eluted with 30% acetic acid. After removal of acetic acid the product was examined by paper chromatography in solvents A, B, C and D, which did not show any new spot in addition to those obtained with the normal enzymic hydrolyzate of the ammonium pectate. The acetic acid-eluate, after being dried, was completely hydrolyzed by refluxing with N-sulfuric acid for 14 hours, giving only galacturonic acid.

Auto-hydrolysis

The water-soluble pectic acid (500 mg.) was dissolved in water (180 ml.) and heated under reflux for 24 hours at 90°C. After concentration, the solution was poured into ethanol and the precipitate recovered by filtration. The filtrate was concentrated and examined by paper chromatography in solvents C, E and F. The precipitate was completely hydrolyzed using 72% sulfuric acid.

Partial Hydrolysis with Oxalic Acid

The water-soluble pectic acid (300 mg.) was dissolved in 0.1N oxalic acid (30 ml.) and heated on the water-bath. Aliquots (3 ml.) were withdrawn as outlined in Table 18. Ethanol was added to each aliquot, when a precipitate appeared which was removed by centrifugation. The supernatant solution was concentrated to remove ethanol, treated with barium carbonate and deionized with Amberlite IR-120(H). The residue, after centrifugation, was washed with ethanol and petroleum ether, dried *in vacuo* over calcium chloride. The product was hydrolyzed with 72% sulfuric acid. This hydrolyzate and the partial hydrolysate were

examined in solvents A, B and E.

Partial Hydrolysis with Formic Acid

The pectic acid (250 mg.) was dissolved in water (15 ml.); 90% formic acid (15 ml.) was added, and the solution was heated on a water-bath. The partial hydrolyzates and the degraded pectic acids were collected in the same way as described in the previous case. The formic acid was eliminated by evaporation with water several times and the sample was refluxed with 0.5N sulfuric acid for about 10 minutes to hydrolyze formate esters. The sulfuric acid was neutralized with barium carbonate, and the solution was filtered through Celite, treated with Amberlite IR-120(H) and examined by paper chromatography in solvents A, B and E.

Partial Hydrolysis with Sulfuric Acid

A similar experiment was carried out using sulfuric acid. The pectic acid (1.0 g.) was dissolved in 1N sulfuric acid and refluxed on the water-bath. Aliquots were withdrawn as shown in Table 21 and treated in the same way as described in the case of the partial hydrolysis with oxalic acid.

Large-Scale Partial Hydrolysis

Purified ammonium pectate (40 g.) was dissolved in water (972 ml.) and concentrated sulfuric acid (28 ml.) was added, after which the solution was heated under reflux on a boiling water-bath for 3.5 hours. After cooling, an equal volume of acetone was added. The precipitate formed was recovered by filtration and washed with acetone:water (1:1).

The filtrate and washings were concentrated, neutralized with barium carbonate, and treated with Amberlite IR-120(H). The degraded pectic acid (18 g.) gave only galacturonic acid on hydrolysis. The partial hydrolyzate was added to the top of a column of Amberlite CG-45 (formate form), anion exchange resin. The column was eluted with water until the eluate gave negative Molisch test. It was then eluted with increasing concentrations (0.001N ---> 2N) of formic acid. Each test tube was qualitatively tested for sugar by staining with o-aminodipehnyl and by measuring the optical rotation. Eight fractions were collected and each of them was examined by paper chromatography in different solvent systems.

The acidic oligosaccharides containing rhamnose were mostly present in one fraction and this was, therefore, resolved into its components using paper chromatography in solvent D.

Since the di- and trigalacturonic acids were not obtained in single fraction and were contaminated also by other sugars, they could not be isolated in a good yield in this way. For further characterization, they were, therefore, isolated in yields of 1.0 g. and 0.8 g., respectively, by treating unfractionated ammonium pectate (purified) (15.0 g. in 1.5 liters of water) with pectinase (3.0 g.) in the same way as described before. Paper chromatography was carried out in solvent B using Whatman No. 3MM filter paper stitched with a wick of No. 1 filter paper.

Characterization of Oligosaccharide (d) [Table 22]

This oligosaccharide was separated from the other oligomers and galacturonic acid by using paper chromatography in solvent D. The

isolated material was further purified by ion exchange chromatography. The solution was added to the top of a column of Dowex 1-X4 (acetate), which was then washed thoroughly with water. The oligosaccharide was eluted with 30% acetic acid. After removal of acetic acid by evaporation with water several times, the aqueous solution was treated with Amberlite IR-120(H) and charcoal, concentrated and dried in vacuo over calcium chloride. Yield, 0.3 g., $[\alpha]_D$ +108°. The oligosaccharide gave a single spot on chromatography in different solvent systems and also on paper electrophoresis, and showed infrared absorptions at the following frequencies (cm^{-1}): 3390 (s), 2970 (m), 2930 (m), 2600 (w), 1740 (s), 1635 (m), 1455 (m), 1425 (m), 1350 (m), 1270 (m), 1235 (m), 1157 (s), 1087 (s), 1065 (s), 1035 (s), 1021 (s), 972 (m), 950-838 (w), 820 (m), 765 (w) and 740 (w).

A portion of the oligosaccharide (50 mg.) was refluxed with dry methanolic hydrogen chloride (1N) and the resulting methyl ester-methyl glycoside was reduced with sodium borohydride. The reduced product was hydrolyzed with N-sulfuric acid, giving galactose and rhamnose.

Another portion (5 mg.) was dissolved in water (1.0 ml.) to which sodium borohydride (5 mg.) was added and kept overnight at room temperature. The excess borohydride was destroyed with acetic acid, and the solution was treated with Amberlite IR-120(H). Boric acid was removed by repeated evaporation with methanol at 45-50°C. The reduced product was completely hydrolyzed with N-sulfuric acid.

Methylation - The oligosaccharide (140 mg.) was dissolved in water (8 ml.) in a three-necked round bottom flask which was placed in water (temp. 20 ± 1). Nitrogen was passed through the system and

vigorous stirring was maintained during the course of the reaction. First, 20 ml. of 30% sodium hydroxide was added. Dimethyl sulfate (10 ml.) was added dropwise very slowly during 2 hours. Two further additions of the reagents were made during another 4 hours, after which the reaction mixture was stirred for two hours more. The solution was heated in the boiling water-bath for 10 minutes, cooled and acidified with sulfuric acid. The aqueous solution was extracted with chloroform (3 x 300 ml.), and the chloroform extract was concentrated to a sirup (78 mg.). When a small quantity of diethyl ether was added crystallization occurred. After recrystallization from a chloroform:ether mixture (1:1), the compound methyl 2-O-(2,3,4-tri-O-methyl-D-galactopyranosyluronic acid)-3,4-di-O-methyl-L-rhamnopyranoside dihydrate had m.p. 66-68°C, $[\alpha]_D$ +98° (c, 0.52 in chloroform), and OCH_3, 39.9%. The infrared spectrum, obtained with a Perkin-Elmer Infracord spectrophotometer, has been presented in Fig. 18.

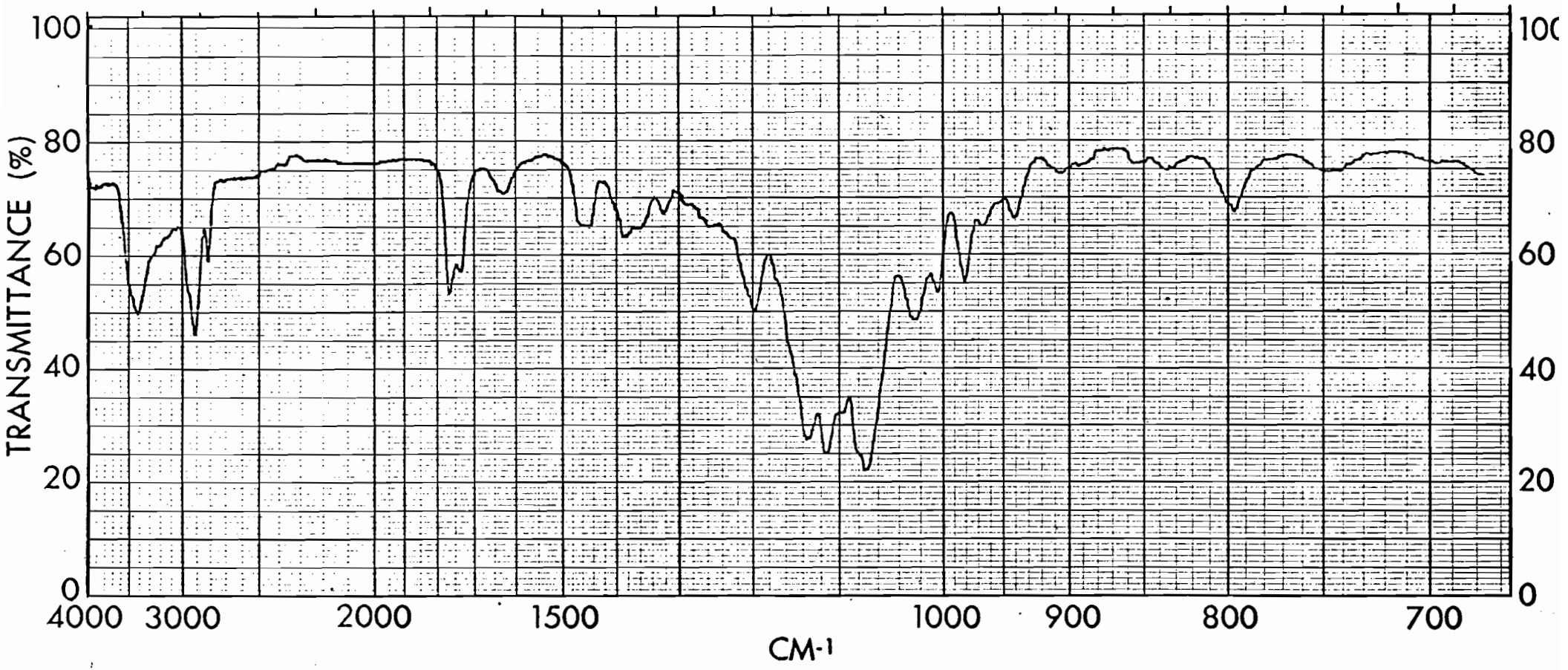
Reduction and Hydrolysis of the Methylated Product

Boron trifluoride-ether complex (4.0 g.) in diglyme (10 ml.) was added dropwise with stirring during one hour to sodium borohydride in diglyme (10 ml.) through which a slow stream of nitrogen was passed, and the liberated diborane was passed into tetrahydrofuran (40 ml.).

Diborane in tetrahydrofuran (1.5 ml.) was added to the methylated oligosaccharide (51 mg.) in tetrahydrofuran (1 ml.). The solution was kept at room temperature for 3 hours, excess of diborane was destroyed by the addition of ethanol and water, the solution was dried, and the residue was repeatedly evaporated with methanol to remove boric acid. The resulting product was hydrolyzed with N-sulfuric acid (refluxed

FIGURE 18

Infrared spectrum for methyl 2-O-(2,3,4-tri-O-methyl-D-galactopyranosyluronic acid)-3,4-di-O-methyl-L-rhamnopyranoside dihydrate


TRANSMITTANCE (%)
100
80
60
40
20
0
4000
3000
2000
1500
1000
900
800
700
CM-1

for 4 hours). The hydrolyzate was recovered in the usual way and then resolved into two components (Table 23) by paper chromatography in solvent H.

TABLE 23

Methylated sugars obtained on hydrolysis of the methylated galacturonosyl rhamnose after reduction with diborane

Fractions	Weight, (mg.)	R_G values in solvent G	H	Sugar on demethylation	Probable assignment
I	12.0	0.67	0.33	galactose	2,3,4-tri-O-methyl-D-galactose
II	10.1	0.85	0.75	rhamnose	3,4-di-O-methyl-L-rhamnose

Characterization of Oligosaccharide (b) [Table 22]

This oligosaccharide was freed from the contaminating oligosaccharide (a) by renewed chromatography in solvent D, treated with Amberlite IR-120(H) and charcoal, and finally obtained as a white powder (89 mg.), which gave single spot on paper chromatography and electrophoresis. It had $[\alpha]_D$ +118° (c, 0.5 in water) and showed infrared absorption bands at the following frequencies (cm^{-1}): 3380 (s), 2940 (w),

2900 (m), 2600 (w), 1735 (s), 1630 (m), 1410-1227 (m), 1148 (s), 1075 (s), 1030 (s), 982 (m), 947-835 (w), 814 (m), 790 (w), 750 (w) and 700 (w).

Partial hydrolysis - The oligosaccharide (15 mg.) was dissolved in 0.2N sulfuric acid (6 ml.) and refluxed for 45 minutes. After neutralization with barium carbonate and treatment with Amberlite IR-120(H), the resulting solution was concentrated and examined in solvent D.

In another experiment the oligosaccharide (15 mg.) was treated with sodium borohydride in aqueous solution for 24 hours, and the reduced product was partially hydrolyzed with 0.2N sulfuric acid. The results obtained on paper chromatography are summarized as follows:

	Sugars (detected on the paper chromatogram)		
	Galacturonic acid	Rhamnose	Galacturonosyl rhamnose
Oligosaccharide	++	++	+
Reduced oligosaccharide	trace	-	+

Methylation - The oligosaccharide (25 mg.) was methylated with dimethyl sulfate and sodium hydroxide in the usual way. The methylated product could not be induced to crystallize and was further treated with silver oxide and methyl iodide. After shaking for two days the sirup (15 mg.) was recovered and completely hydrolyzed with N-sulfuric acid. The hydrolyzate showed two spots, one of which did not move from the starting line in solvent H, and was probably due to a methylated uronic acid, while another spot had R_G 0.76 in solvent H. This was possibly 3,4-di-O-methyl-L-rhamnose.

Characterization of Oligosaccharide (c) [Table 22]

This oligosaccharide was obtained on enzymic hydrolysis of pure ammonium pectate. It was treated with Amberlite IR-120(H) and charcoal and recovered in the usual way. The isolated material gave a single spot on chromatography (R_{GalA} 0.23, 0.60, 0.51 and 0.18 in solvents A, B, C and D, respectively) and on paper electrophoresis (M_G 0.95); $[\alpha]_D$ +154.3 (c, 0.5); decomposes at 125-135°C. The calcium salt had $[\alpha]_D$ +113° (c, 0.5 in N hydrochloric acid). The infrared spectrum of the digalacturonic acid showed absorption at the following frequencies (cm^{-1}): 3410 (s), 2930 (m), 2840 (w), 2750 (w), 1746 (s), 1645 (w), 1420 (m), 1345 (m), 1233 (m), 1158 (s), 1105 (s), 1080 (s), 1060 (m), 1030 (s), 955 (m), 898 (m), 870 (w), 814 (m) and 800 (w).

Conversion to galactobiose - The oligosaccharide (600 mg.) was added to trimethylorthoformate (15 ml.) to which concentrated hydrochloric acid (2 drops) was added. The solid did not dissolve completely on shaking for 12 hours at room temperature. When the mixture was heated to 40°C complete dissolution occurred. The solution was allowed to stand at room temperature for another 12 hours, after which it was neutralized with diazomethane in ether and the resultant solution was evaporated to a sirup, which was dissolved in dioxan (20 ml.). Lithium aluminum hydride (800 mg.) was added slowly and the reaction mixture was refluxed for one hour. After two hours the excess of the hydride was destroyed by the addition of ethyl acetate followed by water. The slurry was filtered, and the filtrate was treated with Amberlite IR-120(H) and 4B (acetate), filtered again, and evaporated to a sirup. The latter was dissolved in 0.1N hydrochloric acid (10 ml.) and left for 20 hours at room temperature, after which it was passed through a column containing

Amberlite 4B (acetate). The eluate was concentrated to a sirup (120 mg.), which on chromatography showed mainly one spot ($R_{galactose}$ 0.19 in solvent E) with a faint spot corresponding to galactose. The latter was removed by paper chromatography in solvent E. The purified product was identical with an authentic sample of an α-(1 --> 4)-linked galactobiose, both chromatographically and ionophoretically, although it could not be induced to crystallize. The disaccharide had $[\alpha]_D$ +175° (c, 0.5), $R_{Galactose}$ 0.39 in solvent I, $R_{Lactose}$ 1.09 in solvent A and M_G 0.5. On complete hydrolysis it gave only galactose.

Preparation of the methyl ester - methyl glycoside of the penta-acetate of digalacturonic acid - The digalacturonic acid (100 mg.) was boiled under reflux with 2% dry methanolic hydrogen chloride for 10 hours. After neutralization with silver carbonate, filtration through Celite, and evaporation, a hard sirupy material was obtained. The product, which was not acidic and did not give any color with o-aminodiphenyl in acetic acid, was dissolved in pyridine (7.5 ml.), and acetic anhydride (2.5 ml.) was added. After 24 hours at room temperature, the solution was poured into ice-water (80 ml.) and the aqueous solution was extracted with chloroform. The chloroform was extracted three times with ice-cold, 10% hydrochloric acid and subsequently three times with a saturated sodium bicarbonate solution. After drying over anhydrous sodium sulfate, the chloroform solution was evaporated to yield a yellow sirup which failed to crystallize; $[\alpha]_D$ +97° (c, 0.8 in chloroform).

Characterization of Oligosaccharide (a) [Table 22]

This oligosaccharide from the unfractionated ammonium pectate, was treated with Amberlite IR-120(H) and charcoal and recovered in the

usual way. It gave a single spot on chromatography (R_{GalA} values 0.04, 0.39, 0.26, 0.05 in solvents A, B, C and D, respectively) and on electrophoresis (M_G 0.97). It had $[\alpha]_D$ +187.2 (c, 0.6), and melted with decomposition at 135-142°C. The calcium salt had $[\alpha]_D$ +149° (c, 0.4 in N hydrochloric acid). The infrared spectrum showed maxima at the following frequencies (cm^{-1}): 3420 (s), 2930 (m), 2650 (w), 2580 (w), 1745 (s), 1640 (w), 1415 (m), 1275 (m), 1230 (m), 1155 (s), 1105 (s), 1080 (s), 1065 (s), 1025 (s), 995 (m), 952 (m), 890 (m), 865 (w), 828 (m) and 800 (w).

Partial hydrolysis - The oligosaccharide (30 mg.) was dissolved in 0.5N sulfuric acid (3 ml.) and heated on the water bath for 45 minutes. The hydrolyzate contained galacturonic acid, digalacturonic acid and some trigalacturonic acid (paper chromatogram).

Methanolysis, reduction, and methylation (100) - The oligosaccharide (600 mg.) was dissolved in dry methanolic hydrogen chloride (1.5% v/v; 50 ml.) and the solution was kept in a stoppered vessel at room temperature for 24 hours. The solution was neutralized with silver carbonate, filtered and evaporated. The resulting substance, which did not give any color with o-aminodiphenyl in acetic acid, was dissolved in water (25 ml.) and borohydride (200 mg.) was added. After 60 hours at room temperature, the reaction mixture was acidified with acetic acid, treated with Amberlite IR-120(H) and evaporated several times from methanol. Finally, the reduced product was methylated with dimethyl sulfate and sodium hydroxide in the same way as described in the case of oligosaccharide (d). The product obtained was further methylated at room temperature with methyl iodide and silver oxide in the presence of Drierite for two days. An aliquot on hydrolysis showed the presence of some acidic methylated sugar.

The methylated product was therefore further reduced with lithium aluminum hydride in tetrahydrofuran and the reduced product was treated with methyl iodide and silver oxide, giving a product with OCH_3, 45.4%. Calcd. for $C_{29}H_{54}O_{16}$: OCH_3, 52.1%.

On hydrolysis of the methylated product (100 mg.) three components were obtained which were separated by paper chromatography in solvent H. One fraction (20 mg.) was identified as 2,3,4,6-tetra-O-methyl-D-galactose (R_G 0.9 and 0.8 in solvents G and H respectively) by its conversion to the aniline derivative, m.p. and mixed m.p. 192-194°C. Another fraction (35 mg.) was identified as 2,3,6-tri-O-methyl-D-galactose (R_G 0.62 and 0.5 in solvents G and H respectively) by its conversion to the corresponding galactonolactone, m.p. and mixed m.p. 97-98°C. The third fraction (21 mg.) had $[\alpha]_D$ +80° (c, 0.8), R_G 0.5 and 0.2 in solvents G and H respectively and M_G 0.31, and did not crystallize. Its aniline derivative could not be induced to crystallize.

REFERENCES

1. M.A. Joslyn, Adv. Food Res., 11, 1 (1962).

2. W.A. Bender, "Industrial Gums, Polysaccharides and Derivatives" (R.L. Whistler and J.N. BeMiller, eds.) pp. 377-432. Academic Press, New York (1959).

3. G.H. Segall and C.B. Purves, Pulp and Paper Mag. Can., 47, No. 3, 149 (1946).

4. E.F. Kurth, Chem. Revs., 40, 33 (1947).

5. C.G. Schwalbe and K.E. Neumann, Cellulosechem., 11, 113 (1930).

6. H.W. Buston and H.S. Hopf, Biochem. J., 32, 44 (1938).

7. K.H. Cram, J.A. Eastwood, F.W. King and H. Schwartz, Dominion Forest Service Circular No. 62, 1947 (Canada), Chem. Abstr., 41, 4643 (1947).

8. Y.P. Chang and R.L. Mitchell, Tappi, 38, 315 (1955).

9. T.J. Painter and C.B. Purves, Tappi, 43, 729 (1960).

10. T.E. Timell, Svensk Papperstidn., 64, 651 (1961).

11. A. Jabbar Mian and T.E. Timell, Tappi, 43, 775 (1960).

12. A. Jabbar Mian and T.E. Timell, Can. J. Chem., 38, 1191 (1960).

13. T.E. Timell, Tappi, 44, 88 (1961).

14. T.E. Timell, Svensk Papperstidn., 64, 748 (1961).

15. T.E. Timell, Svensk Papperstidn., 65, 843 (1962).

16. T.E. Timell, Svensk Papperstidn., 64, 744 (1961).

17. K.V. Ramalingam, Ph.D. Thesis, McGill University, 1963.

18. K.D. Hay and H.F. Lewis, Paper Trade J., 111, No. 25, 39 (1940).

19. V.I. Sharkov, Z.A. Tyagunova and V.N. Kolpina, Lesokhim. Prom., 6, No. 2, 9 (1938); Khim Referat Zhur. 2, No. 3, 123 (1939); Chem. Abstr. 34, 1050 (1940).

20. V.I. Sharkov, A. Girchits and F.A. Sartaniya, Lesokhim. Prom. No. 3, 40 (1939); Khim Referat Zhur. No. 8, 113 (1939); Chem. Abstr. 34, 5273 (1940).

21. V.I. Sharkov and A. Girchits, Lesokhim. Prom. No. 2, 9 (1939); Khim

Referat Zhur. No. 9, 113 (1939); Chem. Abstr. 34, 6324 (1940).

22. E. Anderson and W.W. Pigman, Science, 105, 601 (1947).

23. E. Anderson, L.E. Wise and E.K. Ratliff, Tappi, 37, 422 (1954).

24. W.W. Pigman, E. Anderson and R.L. Leaf, J. Am. Chem. Soc., 70, 432 (1948).

25. T.E. Timell and A. Jabbar Mian, Tappi, 44, 788 (1961).

26. J.P. Thornber and D.H. Northcote, Biochem. J., 81, 449 (1961).

27. J.P. Thornber and D.H. Northcote, Biochem. J., 81, 455 (1961).

28. R.M. McCready and M. Gee, J. Agr. Food Chem., 8, 510 (1960).

29. R.J. Coleman, J.F. Lenney, A.T. Coscia and F.J. Di Carlo, Arch. Biochem. Biophys., 59, 157 (1955).

30. G.O. Aspinall and A. Cañas-Rodriguez, J. Chem. Soc., 4020 (1958).

31. C.M. Martin and F.H. Reuter, Nature, 164, 407 (1949).

32. G.O. Aspinall and R.S. Fanshawe, J. Chem. Soc., 4215 (1961).

33. D.M.W. Anderson and N.J. King, J. Chem. Soc., 5333 (1961).

34. S. Ôno, Bull. School Agr. and Forestry, Taihoku Imp. Univ., 1 (1940); Chem. Abstr., 35, 1766 (1941).

35. C.S. Sato, R.U. Byerrum and C.D. Ball, J. Biol. Chem., 224, 727 (1957).

36. C.T. Bishop, Can. J. Chem., 33, 1521 (1955).

37. E.L. Hirst and J.K.N. Jones, Advances in Carbohydrate Chemistry, 2, 235 (1946).

38. P. Andrews, L. Hough, D.B. Powell and B.M. Woods, J. Chem. Soc., 774 (1959).

39. H.O. Bouveng and H. Meier, Acta Chem. Scand., 13, 1884 (1959).

40. H.O. Bouveng and B. Lindberg, Advances in Carbohydrate Chemistry, 15, 53 (1960).

41. R.W. Bailey and J.B.Pridham, Advances in Carbohydrate Chemistry, 17, pp. 132-134 (1962).

42. A.C. Neish, In K. Kratzl and G. Billek, "Biochemistry of Wood", pp. 82-91, Pergamon Press, New York, 1959.

43. E.L. Hirst, J. Chem. Soc., 70 (1942).

44. C.G. Seegmiller, B. Axelrod and R.M. McCready, J. Biol. Chem., 217, 765 (1955).

45. C.G. Seegmiller, R. Jang and W. Mann, Arch. Biochem. Biophys., 61, 422 (1956).

46. R.L. Whistler and C.L. Smart, "Polysaccharide Chemistry", p. 162, Academic Press, Inc., New York, 1953.

47. E.L. Hirst and J.K.N. Jones, J. Chem. Soc., 1225 (1947).

48. U.K. Sen Gupta and C.V.N. Rao, Bull. Chem. Soc., Japan, 36, 1683 (1963).

49. R.H. Zienius and C.B. Purves, Tappi, 43, 27 (1960).

50. R.H. Zienius and C.B. Purves, Can. J. Chem., 37, 1820 (1959).

51. R.W. Bailey and J.B. Pridham, Advances in Carbohydrate Chemistry, 17, pp. 145-146 (1962).

52. D.M.W. Anderson, A.M. Bews, S. Garbutt and N.J. King, J. Chem. Soc. 5230 (1961).

53. H. Neukom, H. Deuel, W.J. Heri and W. Kündig, Helv. Chim. Acta, 43, 64 (1960).

54. W. Heri, H. Neukom and H. Deuel, Helv. Chim. Acta, 44, 1939 (1961).

55. W.Q. Yean and D.A.I. Goring, Tappi, 47, 16 (1964).

56. W. Heri, H. Neukom and H. Deuel, Helv. Chim. Acta, 44, 1945 (1961).

57. A. Haug, Acta Chem. Scand., 13, 601 (1959).

58. W. Kuhn, Ber., 63, 1503 (1930).

59. T.J. Painter, Can. J. Chem., 37, 497 (1959).

60. O. Perila and C.T. Bishop, Can. J. Chem., 39, 815 (1961).

61. T.E. Timell, Svensk Papperstidn., 65, 435 (1962).

62. D. French and G.M. Wild, J. Am. Chem. Soc., 75, 2612 (1953).

63. J.K. Hamilton and N.S. Thompson, J. Am. Chem. Soc., 79, 6464 (1957).

64. B. Vollmert, Angew. Chem., 61, 329 (1949).

65. B. Vollmert, Macromol. Chem., 5, 110 (1950).

66. H. Neukom and H. Deuel, Chem. and Ind., 683 (1958).

67. J. Kenner, Chem. and Ind., 727 (1955).

68. W.N. Haworth, J. Chem. Soc., 107, 8 (1915).

69. H.C. Srivastava, S.N. Harshe and P.P. Singh, Tetrahedron Letters, No. 27, 1869 (1963).

70. K. Wallenfels, G. Bechtler, R. Kuhn, H. Trischmann and H. Egge, Angew. Chem., 75, 1014 (1963).

71. R.L. Whistler and J.N. BeMiller, Advances in Carbohydrate Chemistry, 13, P. 314 (1958).

72. R. Kuhn, H. Trischmann and I. Löw, Angew. Chem., 67, 32 (1955).

73. F. Smith and A.M. Stephen, Tetrahedron Letters, No. 7, 17 (1960).

74. S.C. McKee and E.E. Dickey, J. Org. Chem., 28, 1561 (1963).

75. H. Deuel, Helv. Chim. Acta, 30, 1523 (1947).

76. T. Takahashi, F. Kasahara and E. Nishide, J. Chem. Soc. Japan, Ind. Chem. Sect., 65, 1420 (1962).

77. J.F. Carson and W.D. Maclay, J. Am. Chem. Soc., 68, 1015 (1946).

78. H. Lineweaver and E.F. Jansen, Advances in Enzymology, 11, 267 (1951).

79. J. Conchie and G.A. Levvy, Biochem. J., 65, 389 (1957).

80. G.O. Aspinall, I.M. Cairncross, R.J. Sturgeon and K.C.B. Wilkie, J. Chem. Soc., 3881 (1960).

81. K.J. Palmer and M.B. Hartzog, J. Am. Chem. Soc., 67, 2122 (1945).

82. K. Wallenfels, Naturwissenschaften, 37, 491 (1950).

83. S.K. Banerjee and T.E. Timell, Tappi, 43, 849 (1960).

84. A. Roudier and L. Eberhard, Bull. Soc. Chim. France, 2074 (1960).

85. R.E. Gill, E.L. Hirst and J.K.N. Jones, J. Chem. Soc., 1469 (1939).

86. L. Hough, J.K.N. Jones and E.L. Hirst, Nature, 165, 34 (1950).

87. R.A. Laidlaw and E.G.V. Percival, J. Chem. Soc., 528 (1950).

88. R.L. Whistler and H.E. Conrad, J. Am. Chem. Soc., 76, 3544 (1954).

89. H. Meier, Acta Chem. Scand., 16, 2275 (1962).

90. C.A. Bunton, T.A. Lewis, D.R. Llewellyn and C.A. Vernon, J. Chem. Soc., 4419 (1955).

91. C. Armour, C.A. Bunton, S. Patai, L.H. Selman and C.A. Vernon, J. Chem. Soc., 412 (1961).

92. J.T. Edward, Chem. and Ind. (London), 1102 (1955).

93. Z.I. Kertesz, "The Pectic Substances", pp. 186-191, Interscience Publishers, Inc., New York, 1951.

94. P. Andrews and J.K.N. Jones, J. Chem. Soc., 4134 (1954).

95. A.M. Stephen, J. Chem. Soc., 1919 (1957).

96. F.S. Smith and R. Montgomery, "The Chemistry of Plant Gums and Mucilages", A.C.S. Monograph No. 141, p. 228, Reinhold Publishing Corporation, New York (1959).

97. H.F. Launer and Y. Tomimatsu, J. Org. Chem., 26, 541 (1961).

98. W.W. Reid, "Methods in Carbohydrate Chemistry", Vol. I, pp. 309-312, edited by R.L. Whistler and M.L. Wolfrom, Academic Press, New York and London, 1962.

99. J.K.N. Jones and W.W. Reid, J. Chem. Soc., 1890 (1955).

100. J.K.N. Jones and W.W. Reid, J. Chem. Soc., 1361 (1954).

101. J. Staněk, M. Černý, J. Kocourek, and J. Pacák, "The Monosaccharides", p. 704, Academic Press, New York and London, 1963.

102. E. Pacsu and S.M. Trister, J. Chem. Soc., 2301 (1940).

103. G.J. Robertson and R.A. Lamb, J. Chem. Soc., 1321 (1934).

104. T.G. Bonner and E.J. Bourne, "Methods in Carbohydrate Chemistry", Vol. II, pp. 206-207, edited by R.L. Whistler and M.L. Wolfrom, Academic Press, New York and London, 1962.

105. T.E. Timell, C.P.J. Glaudemans and A.L. Currie, Anal. Chem., 22, 1916 (1956).

106. J.F. Saeman, W.E. Moore, R.L. Mitchell and M.A. Millett, Tappi, 37, 336 (1954).

107. L.E. Wise, M. Murphy and A.A. D'Addieco, Paper Trade J., 122, No. 2, 35 (1946).

108. T.E. Timell, Svensk Papperstidn., 63, 668 (1960).

109. M. Reintjes, D.D. Musco and G.H. Joseph, J. Food Sci., 27, 441 (1962).

110. P. Kooiman and G.A. Adams, Can. J. Chem. 39, 2423 (1961).

111. E. Pacsu, S.M. Trister and S.W. Green, J. Am. Chem. Soc., 61, 2444 (1939).

112. J.V. Stabin and E.H. Immergut, J. Poly. Sci., 14, 209 (1954).

113. B.H. Zimm and I. Myerson, J. Am. Chem. Soc., 68, 911 (1946).

114. D.J. Bell and G.D. Greville, J. Chem. Soc., 1136 (1955).

115. E.L. Hirst and J.K.N. Jones, J. Chem. Soc., 506 (1946).

116. A.R.N. Gorrod and J.K.N. Jones, J. Chem. Soc., 2522 (1954).

117. Tappi T 209 m-45.

118. T.E. Timell and C.B. Purves, Svensk Papperstidn., 54, 303 (1951).

SUMMARY AND CLAIMS TO ORIGINAL RESEARCH

1. Crude ammonium pectate was obtained from the bark of amabilis fir [Abies amabilis (Dougl.) Forb.] in a yield of 5.4% of the extractive-free bark.

2. The crude product was purified by precipitating calcium pectate and regenerating the ammonium salt. The purified pectin obtained in a yield of 32.7% of the crude product gave on hydrolysis D-galacturonic acid, D-galactose and L-arabinose in a ratio of 85:4:11 and also a trace of L-rhamnose.

3. The purified ammonium pectate was found to be free from any neutral polysaccharide by ion exchange chromatography on diethylamino-ethylcellulose (phosphate form) and also by other fractionation methods.

4. It was discovered that the purified ammonium pectate could be resolved into two fractions by several different methods on the basis of the difference in solubility of the free acids in water. Treatment with Amberlite IR-120(H) exchange resin, acidification with dilute acid and application of electrophoresis-convection technique were useful almost to the same extent to achieve this fractionation. By the addition of an aqueous solution of sodium chloride to the water-solution of the ammonium pectate (unfractionated) it was possible to obtain two similar fractions. Of course, in this case the polysaccharides were recovered as salts.

5. One fraction (water-insoluble), obtained in a yield of 50% of the unfractionated material, gave only D-galacturonic acid on hydrolysis.

Free boundary electrophoresis suggested that this galacturonan was homogeneous.

6. The second fraction (water-soluble), obtained in a yield of 30% of the unfractionated material contained D-galacturonic acid, D-galactose, L-arabinose and L-rhamnose in a ratio of 73:7:18:2. This water-soluble pectic acid could not be resolved further by various fractionation methods. Although this does not constitute an unequivocal proof of chemical uniformity, the neutral sugars were probably integral parts of the pectic acid.

7. Hydrolysis of the polygalacturonic acid with pectinase gave D-galacturonic acid, and its di-, tri-, tetra- and pentamers having $\alpha(1 \rightarrow 4)$-linkages.

8. Successive reduction, methylation and hydrolysis of the polygalacturonic acid gave mainly 2,3,6-tri-O-methyl-D-galactose, a minor amount of 2,3,4,6-tetra-O-methyl-D-galactose and a small quantity of 2,3-di-O-methyl-D-galacturonic acid. The methylated polysaccharide had number average degree of polymerization of 450.

9. The above data showed that the polygalacturonic acid consisted of a linear chain of $(1 \rightarrow 4)$-linked α-D-galactopyranosyluronic acid residues.

10. The water-soluble pectic acid was methylated and hydrolyzed and the O-methyl sugars were resolved into an acidic and a neutral fraction.

11. The acidic part, after reduction, was resolved into 2,3-di-O-

methyl-D-galactose (major part), 2-O-methyl-D-galactose (minor part), 3-O-methyl-D-galactose (minor part), D-galactose (minor part) and a very small amount of 2,3,4-tri-O-methyl-D-galactose.

12. The neutral fraction consisted of 2,3,4,6-tetra-O-methyl-D-galactose and 2,3,5-tri-O-methyl-L-arabinose together with small quantities of 3-O-methyl-L-rhamnose, 2,3,4-tri-O-methyl-D-galactose and an unknown di-O-methyl-L-arabinose.

13. The above data suggested that the water-soluble pectic acid consisted of a backbone of (1 --> 4)-linked α-D-galacturonic acids, which possessed some branching points at C-2 or C-3 of the galacturonic acid and that most of the D-galactose and L-arabinose, but not the L-rhamnose were present as non-reducing end groups. All of the rhamnose and some of the galactose and arabinose residues occurred as inner units, either in the main chain, or, in neutral side chains.

14. Hydrolysis of the water-soluble pectic acid with pectinase gave rhamnose, arabinose, galactose, galacturonic acid, di-galacturonic acid, tri-galacturonic acid and traces of higher oligosaccharides.

15. Partial hydrolysis of the water-soluble pectic acid under different conditions revealed that arabinose was readily removed compared to other neutral sugars and that rhamnose was comparatively stable.

16. Four different acidic oligosaccharides, which originated from the water-soluble pectic acid, were identified.

17. An oligosaccharide containing D-galacturonic acid and L-rhamnose in equimolecular proportions was identified as 2-O-(α-D-galactopyranosyl-

uronic acid)-L-rhamnopyranose.

18. An oligosaccharide containing D-galacturonic acid and L-rhamnose in an approximate ratio of 1:2 was tentatively characterized as O-α-D-galacturonosyl-(1 --> 2)-O-L-rhamnopyranosyl-(1 --> 2)-L-rhamnose.

19. The digalacturonic acid was identified as 4-O-(α-D-galacto-pyranosyluronic acid)-D-galacturonic acid.

20. The trigalacturonic acid was shown to be O-α-D-galacturonosyl-(1 --> 4)-O-α-D-galacturonosyl-(1 --> 4)-α-D-galactopyranosyluronic acid.

21. It was shown by infrared spectroscopy that the D-galactopyranosyl-uronic acid residue in its oligomers and polymers probably forms unstable lactone during concentration of aqueous solution or, more likely, during drying process of the solid or sirup over calcium chloride in vacuo.