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POLYSACCHARIDES OF THE COFFEE BEAN

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Donald Leo Patin, B. S.

The Ohio State University
1964

Approved by

Adviser
Department of Chemistry
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ACKNOWLEDGMENT

The author expresses his appreciation for the interest, counsel, and guidance which Professor M. L. Wolfrom provided during the course of this investigation.

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The author's wife must also be commended for her patience and continued devotion to the goal in mind.
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I. INTRODUCTION AND STATEMENT OF PROBLEM

Previous work in this laboratory led to the collection of much fundamental knowledge of the coffee bean. The green coffee was extracted to leave a basic hard core of polysaccharides. This earlier work led to the quantitization of the sugars in the residual polysaccharides of green coffee. The first phase of this work was to obtain the corresponding fraction of roast coffee.

The largest task confronting this investigator was then to establish the nature of the linkages present in the polysaccharides of the green coffee bean.
II. HISTORICAL

A. Polysaccharides of the Coffee Bean

1. The holocellulose fraction of the coffee bean

The earliest published work concerning the carbohydrates of the coffee bean was that of Payen (1) who reported that glucose (probably sucrose), starch, dextrin, and vegetable acids comprise 15.5% of the green coffee bean. Some time later Schulze and associates (2-4)

(1) A. Payen, Compt. rend., 23, 244 (1846).

partially hydrolysed a polysaccharide component of the green bean with 50% sulfuric acid and isolated D-galactose as the crystalline free sugar and D-mannose as the phenylhydrazone. In his first paper (2) on the green coffee bean Schulze and co-workers found insufficient evidence for pentoses.

In his second paper Schulze (3) worked with the residue from the 5% sulfuric acid hydrolysis. He hydrolysed this with 75% sulfuric acid and although the resulting sirup remained sirupy for
months, D-mannose finally crystallized from it. The presence of glucose in the sirup was shown by oxidizing it to the potassium hydrogen D-glucarate (saccharate). However, Schulze reported that the amount of the potassium salt of D-glucaric acid obtained from the green coffee bean was much less than that obtained from other seeds. Since L-gulose gives the same aldonic acid as D-glucose, Schulze attempted to precipitate all the D-mannose in the sirup as D-mannose phenylhydrazone and then heated the remaining-sirup with phenylhydrazine and obtained a phenyllosasone identical with glucose phenyllosasone. However, mannose also gives the same phenyllosasone. Therefore, it appears that Schulze found very little glucose in the difficultly hydrolyzable fraction of the green coffee bean and did not obtain a confirmatory derivative of it. He did not isolate any galactose or pentose from this fraction.

Ewell (5) reported the finding of a considerable pentose content


in the green coffee bean by the furfural method. This caused Schulze to reinvestigate the green coffee bean and he reported a pentose content of 6.7% by the method of Chalmot and Tollens (6).


In conclusion Schulze reported that the green coffee beans contained, besides sucrose, a soluble carbohydrate portion from alcohol extraction (not investigated), a pentosan, a galactan, and a mannan.
The last three substances were present in the water-insoluble portion of the coffee bean. Gilson (7) claimed to have isolated a mannan by extracting green coffee beans with cuprammonia solution, passing carbon dioxide through the extract and thus precipitating the mannan. However, this procedure was very exacting and there is no record of anyone having repeated it.

For a more complete historical review concerning substances of the coffee bean other than polysaccharides reference is made to the work of Wolfram and Plunkett which is reported in the Ph.D. dissertation of Plunkett (8).

Wolfram and Plunkett isolated and characterized, to some degree, the polysaccharide fractions of the green coffee bean. They fractionated the finely ground bean by successive extractions with 80/20:ethanol/water, 2/l:benzene/ethanol, water, 0.5% aqueous ammonium oxalate, acidified aqueous sodium chlorite, and 10% potassium hydroxide. The residue from the sodium chlorite treatment is called in a general sense (as in the wood chemistry literature) the holo-cellulose of green coffee as it contains a mixture of polysaccharides. The (10% potassium hydroxide)-insoluble portion of the holo-cellulose
shall be designated "holocellulose A." The water-soluble polysaccharide material gave rhamnose (or apiose), and two unidentified carbohydrates on hydrolysis. The ammonium oxalate extract gave galactose, arabinose, mannose, rhamnose (or apiose) and two unidentified carbohydrates on hydrolysis. The (10% potassium hydroxide)-soluble fraction gave rhamnose (or apiose), xylose, arabinose, galactose, a trace of mannose and several unidentified carbohydrates (probably oligosaccharides) on hydrolysis. No crystalline derivatives of these hydrolysis products were isolated. They were identified by paper chromatograms developed simultaneously with known compounds.

The (10% potassium hydroxide)-insoluble residue gave glucose, arabinose, galactose, mannose and one inconclusively identified carbohydrate on hydrolysis. These compounds were identified by paper chromatography with simultaneously run known compounds. D-Mannose was also identified as the crystalline pentaacetate and as the phenylhydrazone. D-Glucose was identified as the crystalline pentaacetate. The amount of D-mannose was estimated as 45.8% by preparation of its phenylhydrazone. The amount of arabinose was estimated as 6.7% by the phloroglucinol method. The arabinose was readily removed by refluxing with 1.5% sulfuric acid.

Matarajan, Khantharaj Urs and Bhatia (9) extracted green and

sugars by paper chromatography without isolating crystalline derivatives.

Natarajan, Khantharaj Urs and Bhatia precipitated the polysaccharides from the water extracts of green and roast coffee with four volumes of alcohol and hydrolyzed them with H hydrochloric acid for five hours. Paper chromatography showed that these fractions contained galactose, arabinose, and mannose but the intensity of the spot for arabinose was low.

According to Thaler (10) roast coffee contains two groups of


materials extractable with cold and hot water, respectively. He fractionated these further by graded alcohol precipitation. The main fraction of the precipitate consisted of glucose, galactose, mannose and arabinose. At lower alcohol concentrations mannose and glucose were predominant; at higher concentrations the galactose content increased.

In another paper Thaler (11) extracted roast coffee with hot water


and precipitated the polymers with Fehling solution. The polysaccharide contained mannose and galactose only and Thaler suggested that it was a galactomannan. It would appear from these two papers by Thaler that he obtained a mixture of water-soluble polysaccharides which he has
been unable to separate into homogeneous polymers. All of this hydrolytic work was conducted on ill-defined mixtures of polysaccharides which many times were contaminated with amino acids, protein, fats, and various other mixtures. At no time were the components really quantitised, until Wolfrom and Plunkett began their studies. Wolfrom and Laver (12) continued the work on the polysaccharide components of the green and roast coffee bean which has been reported along with these efforts in quantitising the non-polysaccharide portions (13). Table 1 is a compilation of the data on the fractionation of green and roast coffee (9,12,13). Wolfrom and Laver hydrolysed the mixture of polysaccharides (holocellulose A) of green coffee by solvation of the very difficultly soluble material in absolute sulfuric acid. The sugars were quantitised by comparative densitometry of the paper chromatographed sirup. This procedure and their results will be further described and compared in the sections of this dissertation concerned with parallel work concerning the hydrolysis and quantitisation of holocellulose A of roast coffee by this author under Professor Wolfrom's direction.


TABLE 1
A Compilation of the Data on the Fractionation of Green Coffee and Roast Coffee (dry weight basis)

<table>
<thead>
<tr>
<th>Fraction extracted * by</th>
<th>Green Coffee b</th>
<th>Roast Coffee b</th>
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<tbody>
<tr>
<td>Ethanol/water:80/20</td>
<td>15.9</td>
<td>16.2</td>
</tr>
<tr>
<td>Benzene/ethanol:2/1</td>
<td>17.4</td>
<td>16.0</td>
</tr>
<tr>
<td>Water</td>
<td>15.7</td>
<td>12.8</td>
</tr>
<tr>
<td>Ammonium oxalate</td>
<td>3.0</td>
<td>3.7</td>
</tr>
<tr>
<td>Acidified sodium chlorite</td>
<td>6.3</td>
<td>8.2</td>
</tr>
<tr>
<td>10% KOH</td>
<td>4.9</td>
<td>8.6</td>
</tr>
<tr>
<td>Alkali-insoluble holocellulose (final residue)</td>
<td>36.8</td>
<td>34.5</td>
</tr>
<tr>
<td>Total</td>
<td>100.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

* Extractions were made successively (from top to bottom).

b Adjusted from a 97.0% actual summation for comparative purposes.
2. **The mannann of green coffee**

Previous to the isolation of the coffee mannann the work on the polysaccharides of the coffee bean had been with the quantitization of the hydrolytic products from polysaccharide mixtures. Wolfrom and Laver were able to isolate the pure mannann of green coffee by extracting the coffee holocellulose A with 10% sodium hydroxide (12). This followed closely the observation by Hamilton, Kircher, and Thompson (14) that mannans were more soluble in sodium hyroxide than


in potassium hydroxide, a finding which was first utilized by Dutton and Hunt (15) in obtaining the mannann of Sitka spruce. Holocellulose A


consisting of 58% D-mannose was extracted with 18% sodium hydroxide and 21% of the starting material was solubilized. Upon acidification (pH 5) of the soluble portion, a white precipitate was formed in a 61% yield. Upon hydrolysis of this material with formic acid a yield of 94% D-mannose and 2% galactose was obtained. The galactose units could not be removed by reprecipitation from sodium hydroxide or by complexing with Fehling solution. The product was considered to be a true mannann by Aspinall's definition of a mannann as a polysaccharide containing 95% or more of D-mannose residues (16). The mannann was
completely methylated (45.4% methoxyl) and hydrolyzed. Paper chromatography showed the primary repeating unit to be a tri-O-methyl sugar.

The completion of the characterization and structural elucidation of this polysaccharide will be described in this dissertation in the appropriate sections as carried out by this author (17).


3. The identification of cellulose

Cellulose, a linear polymer of β-D-glucopyranose is the most abundant of all naturally occurring organic substances and constitutes a third of all vegetable matter. Hence, it exists in far greater quantity than any other polysaccharide (18). It is the main constituent of cell walls of plants and serves therein as the primary structural element. Probably cellulose has the largest molecular weight
of all polysaccharides and is highly resistant to attack by micro-
organisms and chemicals.

Recognition of this polysaccharide as a chemical entity was made
by the distinguished French agriculturist Payen (19). He was the

(19) A. Payen, Compt. rend., 8, 169 (1839).

first to note that it was the fundamental uniform constituent of all
plant cell walls and thus very fittingly described it as cellulose.
Although world wide recognition of Payen's very fundamental classical
work with cellulose and in other areas was late forthcoming, his name
is applied to the annual award given by the Division of Cellulose,
Wood, and Fiber Chemistry of the American Chemical Society.

The purest natural cellulose is the cotton fiber (cotton Linters)
which on the dry basis consist of about 98% cellulose, 1% protein,
0.5% xylan and wax and pectic substances. Wood contains 40-50%
cellulose and is the most important commercial source.

Characteristic tests for cellulose include hydrolysis to D-
glucose in high yield, acetylation to α-cellobiose octaacetate, cupram-
monia solubility, x-ray powder diffraction pattern and others. A
test of this type does not stand by itself but when collected together
constitute a body of information which cannot be refuted. Unfortunat-
ely the presence of cellulose in the coffee bean was overlooked although
holocellulose A contains 18% D-glucose as a polysaccharide (13). The
mixture of polysaccharides therein failed to solvate with cuprammonia
solution probably due to the influence of the encrusting mannan (17).
As described in this dissertation cellulose was finally isolated in a relatively pure state by the use of the selectivity of formic acid in hydrolyzing the other polysaccharides at a faster rate contained in the holocellulose of green coffee (20).


B. Arabinogalactans

1. Occurrence and early investigations

The occurrence of arabinogalactans in nature is quite widespread in the plant kingdom. In order of importance among polysaccharides the distribution of arabinogalactans would be less than that of cellulose, xylans, starch, and arabans. These substances occur most commonly in wood type plants and the presence of a water-soluble, highly branched arabinogalactan in the wood of conifers appears to be a general characteristic of these trees. Most species, however, contain only small amounts of this type of polysaccharide. Larger amounts, however, have so far been found to occur only among members of the Genus Larix (21), where sometimes as much as 25% of the wood might consist of an


arabinogalactan. Some confusion exists in the literature, for example the arabinogalactan of Larix has been referred to as β-galactan, galactan, arabogalactan, and arabinogalactan (22). Plant
arabinogalactans are in general complex, highly branched materials which vary in galactose to arabinose ratio from about 3:1 to 6:1. Their molecular weights vary a great deal, probably being a function of the extraction method employed. Arabinogalactans are often present in the gums and gum exudates of many types of plants but this review will be limited to a discussion of those found only in the woody portions of trees and in maple sap.

The first mention of a water-soluble polysaccharide in larch was made in 1898 (23). What was undoubtedly an arabinogalactan was referred to as a galactan. This was obtained by extraction of larch-wood with water (24). Wise and co-workers carried out investigations on the nature of the arabinogalactans in western larch (Larix occidentalis) (25,26), tamarack (Larix laricina) (27), and European


(23) H. Trimble, Am. J. Pharmacol., 70, 152 (1898).


larch (Larix decidua) (28). White was responsible for a series of
brilliant investigations on the western larch arabinogalactan and
suggested a structure which, although not completely correct, is in
its essential features the same as that currently accepted (29).

With the advent of new chromatographic methods it was possible to
study the structures of these substances in greater detail. This later
work revealed that some larch species contain two closely related
types of arabinogalactans which are usually referred to as A and B
(21). Mosimann and Svedberg separated larch arabinogalactan into two
widely different molecular weight components (m.w. 16,000 and 100,000)
by ultracentrifugal techniques (30). The two-component aspect has not

been found with the arabinogalactan from European larch (31) and
tamarack (32).

Chem., 25, 184 (1933).

(28) F. C. Peterson, M. Maughan, and L. E. Wise, Cellulosechem.,
15, 109 (1934).

(29) E. V. White, J. Am. Chem. Soc., 63, 2871 (1941); 64, 302,
1507, 2838 (1942).

The isolation of larch arabinogalactan in good yields by extracting the fully lignified wood with water is unique among wood polysaccharides in this respect. The others must be extracted from de-lignified wood and with solvents of much greater swelling power. As shall be pointed out later this ease of solubility is true also for the arabinogalactan found in the coffee bean. Lystad-Borgin (33) isolated an arabinogalactan of western larch from the heartwood,


in 8.4% yield, which seemed to be homogeneous, and one from the sapwood, in 0.9% yield, which could be resolved into two components similar in molecular weights to those obtained by Mosimann and Svedberg (30). Bouveng and Lindberg (34) undertook a systematic investigation of


western larch and were able to resolve the material obtained by water extraction of the heartwood by electrophoresis on glass fiber sheets in borate buffer. The more mobile component, arabinogalactan A, is generally obtained in smaller amounts than B and the various yields
from many larches and their general properties are presented by Timell (21). Because of the complicated nature of these polysaccharides the structural assignments of four species shall be presented individually.

2. Western larch arabinogalactan

In his classical study of the western larch (Larix occidentalis) arabinogalactans (working with the mixture of A and B) White (29) obtained upon methylation and hydrolysis 2,4-di-O-methyl-D-galactose, 2,3,4-tri-O-methyl-D-galactose, 2,3,4,6-tetra-O-methyl-D-galactose and 2,3,5-tri-O-methyl-L-arabinose in a mole ratio of 3:1:3:2. Part of the arabinose groups were removed by mild hydrolysis and the new polysaccharide was remethylated and hydrolyzed to give the 2,4-di-, 2,3,4-tri-, 2,4,6-tri-, and 2,3,4,6-tetra-O-methyl-D-galactose in a mole ratio of 2:1:1:2. This made it quite evident that with the removal of one arabinose residue, there was also lost one 2,4-di-O-methyl-D-galactose while one mole of 2,4,6-tri-O-methyl-D-galactose was formed. This clearly places the attachment of the L-arabinose unit on C-6 of a (1→3)-linked D-galactose unit.

Partial methanolysis of the fully methylated polysaccharide yielded the disaccharide 6-O-(2,3,4,6-tetra-O-methyl-β-D-galactopyranosyl)-2,3,4-tri-O-methyl-D-galactopyranoside and the corresponding compound without the C-3 methyl group in the galactopyranoside moiety. Thus the final structure suggested by White on the basis of this and other evidence is given in Figure 1.

Probably the most thorough investigation of a wood polysaccharide
FIGURE 1. -- THE STRUCTURE OF THE ARABINOGALACTAN OF WESTERN LARCH (Larix occidentalis) (E.V. WHITE)
(except for cellulose) was that taken up by Bouveng and Lindberg concerning the western larch (34-39). In a preliminary study (37) of the

unfractionated material they were able to isolate 3-2-β-L-arabinopyranosyl-L-arabinose. The occurrence of this disaccharide proved that not all the arabinose is present in the furanose form as assumed by White (29).

Bouveng and Lindberg then studied arabinogalactan A (the fractionated material) by classical methylation techniques (34). They were able to detect some methylated sugars that previously had not been discovered by White. The isolation of 2-α-methyl and 2,6-di-α-methyl-D-galactose indicated the occurrence of doubly branched units and of (1 → 4)-linkages respectively. As would be expected from their isolation of 3-2-β-L-arabinopyranosyl-L-arabinose, 2,5-di-α- and 2,3,4-tri-α-methyl-L-arabinose were also identified. They were also able to estimate that two thirds of the arabinose was present as terminal L-arabinofuranose residues.

Following White's lead Bouveng subjected arabinogalactan A to a mild hydrolysis and all the arabinose residues were removed (36). The product from this treatment could be resolved into two components
by electrophoresis and a subsequent methylation study proved that both residues (containing only galactose) carried the same linkages but one was more highly branched. Their results confirmed White's (29). The easily hydrolysed units isolated in their first work (37) were probably attached by (1 - 6)-linkages as White had assumed. This evidence indicates that arabinogalactan A is homogeneous.

Later studies on both arabinogalactans of western larch employing periodate oxidations and Barry degradations (oxidation with periodate, followed by treatment with phenylhydrazine-acetic acid) (40) closely


confirmed the earlier methylation studies on A and those concerning B (38) that both polysaccharides are very similar.

Bouveng concludes that the main repeating unit is the (1 - 3)-β-D-galactopyranose, that almost all of the C-6 positions carry substituents, each side-chain carrying an average of two sugar residues. The arabinose is present as terminal L-arabinofuranose residues, 3-Ω-Ω-L-arabinofuranosyl-L-arabinofuranose, and perhaps in units of three residues. More evidence is needed before deciding on (1 - 4)-linkages. Therefore this work of Bouveng and Lindberg (34-39) further contributes to the structure proposed by White (Fig. 1) and the linkage sequences shown in Figure 2 represent these efforts.

3. European larch arabinogalactan

The arabinogalactan of European larch (Larix decidua) was unfortunately referred to as ε-galactan in the early literature (21).
FIGURE 2. -- FURTHER STRUCTURAL INFORMATION CONCERNING THE WESTERN LARCH ARABINO GALACTAN
(Larix occidentalis) (BOUVENG AND LINDBERG)
Campbell, Hirst, and Jones (41) fractionated the methylated polysaccharide and obtained what they thought was the methylated product of a galactan. Hydrolysis yielded $2,4$-di-$Q$-, $2,3,4$-tri-$Q$-, and $2,3,4,6$-tetra-$Q$-methyl-D-galactose indicating a branched structure. The authors believed they were dealing with a mixture consisting of galactan and arabinogalactan, or an araban and a second galactan. Later investigations did not confirm these conclusions (31,42). In addition to the galactose derivatives found earlier (41), the latter workers (31,42) obtained in equimolar quantities, $2,5$-di-$Q$-methyl and $2,3,4$-tri-$Q$-methyl-L-arabinose. Only traces of $2,3,5$-tri-$Q$-methyl-L-arabinose were found indicating a virtual absence in this case of this sugar in the terminal furanose form.

Barry degradation of the arabinogalactan gave a resistant polysaccharide (characteristic of the $(1 \rightarrow 3)$-linkage) which on partial hydrolysis yielded $3$-$Q$-β-D-galactopyranosyl-D-galactose. This information coupled with that from the methylation procedure involves the galactose in a major repeating sequence of $(1 \rightarrow 3)$-β-D-galactopyranose units with a $(1 \rightarrow 6)$-β-D-galactopyranose disaccharide on its C-6 position. This accounts for the equimolar amounts of the di-, tri-, and tetra-$Q$-methyl sugars. Further studies were needed to locate
the L-arabinose residues (42) and this information was obtained by employing oxidative methods along with partial hydrolysis. The results indicated that the major portion of arabinose was attached directly to the C-6 position of the galactose backbone while the remainder was attached to the C-6 position of the galactose side-chains. The results of these investigations on European larch then lead to the structure proposed in Figure 3.

4. **Tamarack arabinogalactan**

The arabinogalactan present in the wood of the tamarack (Larix laricina) has been studied extensively by Adams and co-workers (31,43,44). The water-soluble polysaccharide was considered to be homogeneous on the basis of its behavior on fractional precipitation, ultracentrifugation and boundary electrophoresis. Methylation studies were followed by an exhaustive partial hydrolysis program (43). Nine oligosaccharides were isolated in pure form by chromatography on cellulose carbon-Celite columns and a fraction collector. The disaccharides were 3-O-β-L-arabinopyranosyl-L-arabinose, 6-O-β-D-galactopyranosyl-D-galactose and 3-O-β-D-galactopyranosyl-D-galactose. The tri- and tetrasaccharides all involved mixed β-D-(1 → 3)- and β-D-(1 → 6)-linkages. The structural picture which may be built of the polysaccharide from this information is presented in Figure 4.

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FIGURE 3 - THE STRUCTURE OF THE ARABINOGLACTAN OF EUROPEAN LARCH (Larix decidua)
FIGURE 4. — THE STRUCTURE OF THE ARABINOGALACTAN OF TAMARACK (*Larix laricina*)
This structure differs from those previously suggested for similar arabinogalactans for it contains both β-D-(1→3)- and β-D-(1→6)-linked residues in the galactose backbone as well as β-(1→3)-linked terminal side-chains of D-galactopyranose. In later work (44) the presence of D-glucuronic acid was detected in about a 2% concentration.

In summary it is clear that the three arabinogalactans discussed up to this point are very similar structurally. The agreement between workers is quite close and very surprising when considered in the light of the complexity of the molecules and the experimental difficulties encountered. All have a backbone of (1→3)-β-D-galactopyranose residues and one (tamarack) has some (1→6)-linkages scattered through its backbone. The nature of all side chains is not fully understood but the majority seem to contain an average number of two (1→6)-linked β-D-galactopyranose residues while others consist of 3-O-β-L-arabinopyranosyl-L-arabinofuranose residues. In addition a few terminal residues of L-arabinofuranose are present and some evidence exists for double branching and some (1→4)-linked galactose units.

5. **Maple sap arabinogalactan**

Adams and Bishop (45) state that one of the reasons for studying non-cellulosic polysaccharides is that a knowledge of their structures may help in elucidating the modes of formation and physiological roles of these materials in the plants. To them it seems possible that a

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physiologically active material like wood sap might be rich in products of transglycosylation reactions, and a comparison of these non-cellulosic polysaccharides contained therein with those isolated from the wood itself should be of interest. For this reason the maple sap arabinogalactan (Acer saccharum) is included along with the three from wood in this historical survey.

The arabinogalactan was isolated in yields varying from 7.5 to 17.0 mg. per 100 ml. of maple sap after removal of the sucrose and other impurities by dialysis. The polysaccharide was electrophoretically pure and contained residues of D-galactose, L-arabinose and L-rhamnose in molar ratios of 5:4:5:5 respectively. On methylation and hydrolysis they obtained 2,4-di, and 2,4,6-tri-O-methyl-D-galactose, 2,3,4-tri-O-methyl-L-rhamnose, 2,4-di and 2,3,5-tri-O-methyl-L-arabinose. Naturally the presence of rhamnose makes this polysaccharide quite different but the large amount of arabinose and its low rotation (−41°) is unusual also. This arabinogalactan (Figure 5) shares its β-D-(1→3)-linked backbone with the softwood polysaccharides but in other structural details differences are quite evident and it is doubtful that any biogenetic relationship exists between the two types. The authors point out that the existence of rhamnose residues is a characteristic of plant gum polysaccharides in general.
FIGURE 5 - THE STRUCTURE OF THE ARABINOGALACTAN OF MAPLE SAP (Acer saccharum)
III. EXPERIMENTAL

A. Hydrolysis and Quantitative Assay of the (10% Potassium Hydroxide)-Insoluble Holocellulose (Holocellulose A) of Roast Coffee

1. Total hydrolysis of holocellulose A of roast coffee

Holocellulose A of roast coffee, prepared by Wolfram and Laver (13), was used in these experiments.

Sulfuric acid was prepared by mixing 50 ml. of fuming sulfuric acid with 45 ml. of concentrated sulfuric acid. Upon further addition of 15 ml. of concentrated sulfuric acid in 3-ml. aliquots the freezing point of \(-10^\circ\) was reached.

To a 0.9888 g. sample of the (10% potassium hydroxide)-insoluble holocellulose was added 25 ml. of anhydrous sulfuric acid in a three-necked flask submerged in a sodium chloride, ice-water bath at \(-10^\circ\). The mixture solidified at this temperature and was allowed to warm to \(3^\circ\) over a period of 8 hr. At this time approximately 80% of the material was in solution and had turned dark brown in color. Sixteen hours later the material was in solution with the exception of a few particles which were washed down with 3 ml. of acid. A power stirrer was inserted and the mixture was frozen at \(-8^\circ\) to eliminate local heating when the first drops of water were added. Water was allowed to drop at a rate of 1 ml. per min. and the temperature did not rise above \(5^\circ\). After 350 ml. had been added in this manner, a dilution was made to 1500 ml. total volume to give a 3% sulfuric acid concentration.
Refluxing was started and the hydrolysis was followed by using reducing values as obtained by the Somogyi copper reduction method (46). After 12 hr. the hydrolysis was complete and the solution was neutralised to litmus using saturated barium hydroxide solution. The barium sulfate was removed by filtration, the solution concentrated under reduced pressure and refiltered (several times) to remove most of the inorganic salt. Throughout these processes the solution was kept slightly acid to methyl red. The solution was concentrated to a sirup and refluxed with methanol for 3 hr. The remaining inorganic salts were removed by filtration, the methanol evaporated under reduced pressure and the sirup diluted to 200 ml.

2. The quantitative estimation of constituent sugars

Quantities of the hydrolysate ranging from 0.050 ml. to 0.075 ml. were applied to paper chromatograms along with a known solution (containing the constituent sugars) and were developed according to the procedure of Quick (47). The chromatograms were air-dried, sprayed with aniline phthalate reagent, and heated in an oven at 100-115° for 15 min. The densities of the resulting spots were read as quickly as possible by a densitometer (Photovolt Electronic Densitometer, Model 5W, Photovolt Corp., New York, N. Y.) following closely the procedure of McFarren, Brand, and Rutkowski (48), as


used by Wolfram and Laver (12) on the (10% potassium hydroxide) insoluble holocellulose of green coffee hydrolysate. The results obtained were as follows: mannose, 51.4%; glucose, 17.7%; galactose, 11.9%; arabinose, 2.9%; for a total yield of 84%.

The total solids content of the hydrolysate was established by drying 10-ml. aliquots of the hydrolysate on a weighed amount of Celite (previously dried to constant weight) at 45°C. These results indicated a total solids content of 0.852 g. corresponding to a yield of 86%.

3. **Hydrolysis of holocellulose A of roast coffee with 2.5% sulfuric acid**

The method developed by Wolfram and Plunkett (8) was used for this hydrolysis with some modifications. A 0.1097 g. sample of holocellulose A was added to 25 ml. of boiling 2.5% sulfuric acid and refluxed for 20 min. The hydrolysate was treated and chromatographed in the same manner as described previously for the anhydrous sulfuric acid hydrolysis and a yield of 6.7% of arabinose was obtained. This figure of 6.7% for arabinose, combined with the yields reported earlier for the other three constituent sugars, gives a total yield of 87.6% for the two hydrolyses of holocellulose A of roast coffee. (See Table 2.)
B. The Structural Elucidation of the Mannan of Green Coffee

1. Preparation of known tri-\(\alpha\)-methyl-D-mannopyranoses

Because of difficulties encountered by Wolfrom and Laver (13) in isolating a crystalline derivative of 2,3,6-tri-\(\alpha\)-methyl-D-mannopyranose (tentatively identified by paper chromatography) it seemed advisable to continue this work on the structural elucidation of the mannans of green coffee by preparing the two most likely tri-\(\alpha\)-methyl-D-mannopyranoses.

(a) Preparation of 2,3,6-tri-\(\alpha\)-methyl-D-mannopyranose

An amount of 5.0 g. of the (7% potassium hydroxide)-insoluble portion of the ivory nut (Phytelephas macrocarpa) generally designated as "Mannan A" (49), was added to 150 ml. of 10% sodium hydroxide solution and the mixture stirred for 2 hr. resulting in a nice solution. To this solution was added 18 g. of powdered solid sodium hydroxide followed by the dropwise addition of 200 ml. of 30% sodium hydroxide and 150 ml. of dimethyl sulfate (50). The methylation reaction was started at ice-bath temperature and after 3 hr. it was allowed to come to room temperature. The reaction foamed considerably and 50 ml. of acetone was added to prevent the foaming. Throughout the addition of the reagents the reaction mixture was very strongly stirred. After


stirring 24 hr. an additional 10 g. of sodium hydroxide and 50 ml. of dimethyl sulfate was added. Following a total reaction time of 72 hr. the reaction mixture was dialyzed (Visking cellophane casing) against distilled water until neutral. The partially methylated product was isolated by evaporation of the non-dialysables and lyophilisation. This product did not prove to have good tetrahydrofuran solubility so it was remethylated in the same manner. After the second methylation the material was soluble in acetone and tetrahydrofuran. Commercial tetrahydrofuran was dried over potassium hydroxide pellets for several days and then distilled at 64-65°C from potassium hydroxide pellets.

The method of Falconer and Adams (51) was used to complete the


methylation. The lyophilized product of the second methylation was taken up in 400 ml. of dry tetrahydrofuran and although the material appeared to dissolve, the solution remained slightly cloudy. The reaction was stirred strongly and 40 g. of powdered sodium hydroxide was added, followed by the dropwise addition of 50 ml. of dimethyl sulfate and 30 ml. of acetone. After 24 hr. the same amount of reagents was added. Throughout the reaction, additional amounts of tetrahydrofuran were added to maintain fluidity. A heavy precipitate of sodium methyl sulfate was formed during the reaction. The reaction mixture was partly neutralized with hydrochloric acid in an ice-bath and was then dialysed against running water for 3 days. The non-dialyzables were
concentrated under reduced pressure to a heavy, cloudy suspension. A portion of the suspension showed good chloroform solubility. Although this is hardly a positive test for full methylation it was only necessary to isolate a small portion of authentic 2,3,6-tri-\(\alpha\)-methyl-manno-pyranose from the thrice methylated ivory nut mannan.

The hydrolysis of the methylated ivory nut mannan was carried out by following the "formic acid procedure" of Wolfrow and Laver (13). The thrice methylated suspension was dissolved in 300 ml. of 90.6% formic acid and heated on a steam bath for 3 hr. The formic acid was removed under reduced pressure and several successive portions of water were added and subsequently evaporated in an effort to remove all traces of formic acid. The sirup was dissolved in 100 ml. of 0.5 M sulfuric acid and heated on a steam bath for 4 hr.

The hydrolysate was decolorized with carbon, and yielded a clear yellow sirup which was neutralized with Duolite A-4 ion-exchange resin. The concentrated sirup was developed on Whatman No. 3 filter paper with butanone-water aseotrope (52) and the spots were indicated with aniline phthlate spray reagent. The areas corresponding to the tri-\(\alpha\)-methyl sugar were excised and eluted with water. The eluate was concentrated to a sirup under reduced pressure.

A portion (530 mg.) of the sirup was dissolved in pyridine (50 ml.) and \(p\)-nitrobenzoyl chloride (2.6 g.) was added (53). The reaction

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mixture was kept at 60-70° for 30 min. and was then maintained overnight at room temperature. It was neutralized with saturated sodium bicarbonate solution until effervescence ceased. The solution was then extracted with three 100-ml. portions of chloroform and dried with magnesium sulfate which was removed by filtration. The chloroform was evaporated under reduced pressure; the crystalline residue was dissolved in hot methanol and recrystallised twice from methanol, washed with cold methanol, and dried at 50° for 3 hr.; m.p. 185-186°. Pure material was obtained on two more recrystallisations from methanol; m.p. 190-191°, [reported (53) m.p. 190-1°]. The material obtained was authentic 2,3,6-tri-O-methyl-D-mannopyranose 1,4-bis(p-nitrobenzoate).

(b) **Attempted preparation of 2,3,4-tri-O-methyl-D-mannopyranose**

In anticipation of the need also of 2,3,4-tri-O-methyl-D-mannopyranose a synthesis was carried out as follows. Crude levoglucosan (1,6-anhydro-D-glucopyranose) (33 g.) was dissolved in 35% sodium hydroxide (450 ml.). To this heavily stirred mixture was added dimethyl sulfate (180 ml.) dropwise over a period of 4 days (50). The excess dimethyl sulfate was destroyed by heating at 80° for 15 min. The solution was made neutral (to litmus) with sulfuric acid. The solids were removed by filtration, washed heavily with chloroform and the filtrate was extracted (four times) with chloroform. The chloroform phases, upon separation, contained a gelatinous solid.
mixture was heated to boiling and hot ethanol was added to precipitate the inorganic salts which were then removed by filtration and washed with chloroform. The chloroform phase was then evaporated under reduced pressure to a dark brown sirup (25 ml.). To this sirup was added 30% sodium hydroxide (40 ml.) and dimethyl sulfate (30 ml.) dropwise under heavy stirring over a period of 24 hr. Additional amounts of sodium hydroxide (40 g., powdered, and 70 ml., 30%) along with dimethyl sulfate (60 ml.) were added periodically over the next 3 days. This methylation reaction was then processed in the same manner as the first, and the resulting chloroform extract was dried with magnesium sulfate. The drying agent was removed by filtration and the chloroform was removed by evaporation under reduced pressure. To the resulting sirup was added five (200 ml.) portions of absolute ethanol. These were evaporated to sirups each time, removing all traces of chloroform. Upon cooling of the sirup and addition of authentic material, the tri-0-methyllevoglucosan crystallized. The material was recrystallized from chloroform, ethanol, and Skellysolve B. This operation was followed by three recrystallizations from ether and yielded 17.5 g. of 1,6-anhydro-2,3,4-tri-0-methyl-β-D-glucopyranose, m.p. 55-57°.

A portion (5.0 g.) of the tri-0-methyllevoglucosan was hydrolyzed by refluxing in 7% hydrochloric acid (300 ml.) for 4.5 hr. The hydrolysate was neutralized with Duolite A-4 ion-exchange resin and evaporated under reduced pressure to a sirup. A portion of this sirup was loaded on paper chromatograms, developed with ethyl
acetate, acetic acid, and water (9:2:2 v/v/v.), and sprayed with aniline phthalate indicator (47). One single intense spot appeared.

A portion (2/3) of the sirup was made up to a volume of 500 ml. in 0.035 M barium hydroxide solution and stirred with a magnetic stirrer. This is the method of Wolfson and Lewis (54) to effect the


epimerization of the 2-Q-methyl group of an aldose. The reaction was followed by the following observed rotations: starting time, 0.168°; 2 days, 0.126°; 5 days, 0.114°; 7 days, 0.113°. Throughout the epimerization a thin layer of toluene was kept over the reaction mixture. As 2,3,4-tri-Q-methyl-D-mannopyranose was not encountered in the mannan of green coffee structural elucidation this experiment did not have the usefulness which was anticipated. Some time later, however, a gas chromatographic examination of the methanolyzate of the above sirup revealed the mannone derivative in a concentration equal to about one-fourth that of the glucose derivative. The chromatographic details will be described in a later section.

2. Hydrolysis and chromatographic investigation of the fully methylated mannan of green coffee

Although additional fully methylated mannan had to be prepared, the procedures used followed quite closely those described in the preceding section and the product obtained was identical to that prepared by Wolfson and Laver (13), hence additional detail will not be presented.
The hydrolysis of the per-methylated mannan followed the "formic acid procedure" described earlier. The sirup so obtained was chromatographed by two solvent systems which established the homogeneity of the tri-2-methyl sugar.

One of the possible linkages was eliminated by the use of the following system. A solution consisting of 14.9 g. boric acid, 8.0 g. sodium hydroxide, and 2.0 liters of water was prepared. The above sirup was loaded on Whatman No. 3 filter paper alongside equivalent concentrations of tetra-2-methyl-D-glucose and D-glucose. The chromatogram was then developed electrophoretically at 700 volts with the above borate buffer solution at pH 9.2. The results showed no movement of the tri-2-methylmannose spot leaving it stationary alongside that of the tetra-2-methyl-D-glucose while the D-glucose moved very well. This clearly indicates the lack of the open 1,2 dihydroxyl function (the only one prone to borate complexing in 2-methyl sugars) which eliminates the possibility of the (1→2)-linkage.

The sirup was again paper chromatographed and developed with butanone-water azeotrope (52). After extensive development and indication with aniline-phthalate the main spot showed no separation. This solvent system has been used previously by Smith and co-workers (53) to separate all of the possible tri-2-methyl-D-mannose derivatives with the exception of 3,4,6-tri-2-methyl-D-mannose. This possibility was eliminated by use of the borate-buffered electrophoretic work. The tri-2-methylmannose involved has now been proven to be chromatographically pure.
3. **Characterisation of 2,3,6-tri-O-methyl-D-mannopyranose**

(a) **The isolation of 2,3,6-tri-O-methyl-D-mannono-1,4-lactone**

The tri-O-methyl-D-mannopyranose sirup was obtained by developing the methylated mannan hydrolysate chromatographically (Whatman No. 3 filter paper and ethyl acetate, acetic acid, and water) (9:2:2 v/v/v), and excising the areas corresponding to the tri-O-methyl sugar, eluting with water, and concentrating. To the sirup (100 mg.) was added water (7 ml.) and bromine (2.5 ml.) followed by vigorous shaking for 4 hr. (55). The solution was stored for 10 days in the dark at 25°, at which time chromatographic investigation failed to indicate a reducing sugar.

The solution was aerated, neutralized with silver carbonate, filtered, and the filtrate and washings concentrated to 100 ml. A crystal of lead tetraacetate was added (to prevent colloidal silver sulfide formation) and the solution was saturated with hydrogen sulfide. The silver sulfide was removed by filtration, the clear solution concentrated, passed through Amberlite IR-120, and evaporated under reduced pressure to dryness. The residue was dissolved in 50 ml. of ether and upon slow evaporation a yellow precipitate was recovered. The filtrate was concentrated to dryness, the residue dissolved in benzene and hexane (petroleum ether, b.p. 60-68°) added. Nucleation caused immediate crystallisation affording 2,3,6-tri-O-methyl-D-mannono-1,4-lactone; yield 17 mg., m.p. 79.5-81° unchanged after one recrystallisation.

reported m.p. 84-85° (56). The x-ray powder diffraction data yielded

Soc., 1278 (1948).

conclusive evidence according to the following criteria (to be used throughout), interplanar spacing, \( \AA \); CuK\(_x\) radiation; relative intensity, estimated visually: s, strong; m, medium; w, weak; v, very.

First three strongest lines are numbered (1 strongest). These were identical with those of authentic 2,3,6-tri-\( \delta \)-methyl-D-manno-1,4-lactone kindly furnished by Professor J. K. N. Jones: 10.98 s (3), 8.36 m, 7.15 vs (1), 6.63 m, 5.94 s, 5.10 w, 4.60 vs (2), 4.35 s, 4.16 m, 3.96 s, 3.85 s, 3.52 s, 3.30 s, 3.26 s, 2.88 m, 2.69 m, 2.36 m, 2.25 m, 1.96 w.

For comparative purposes the x-ray powder diffraction data were measured of authentic 2,3,4-tri-\( \delta \)-methyl-D-manno-1,5-lactone (which was kindly furnished by Professor J. K. N. Jones): 11.86 vs (2), 9.36 vs (1), 7.73 s, 6.15 w, 5.84 w, 5.10 m, 4.83 m, 4.57 s, 4.56 s, 4.18 s, 3.87 m, 3.60 w, 3.45 w, 3.40 vs (3), 3.21 s, 3.03 s, 2.91 v, 2.80 v, 2.72 w, 2.51 w, 2.47 w, 2.36 w.

(b) The isolation of 2,3,6-tri-\( \delta \)-methyl-D-manno-pyranose 1,4-bis(p-nitrobenzoate)

As described earlier in more detail (in the section concerning this known compound) the following procedure was used. A portion (150 mg.) of the isolated tri-\( \delta \)-methyl-D-manno-pyranose sirup was dissolved in pyridine (10 ml.) and p-nitrobenzoyl chloride (700 mg.) was
added (53). The reaction mixture was kept at 60-70° for 30 min. and was then maintained overnight at room temperature. It was neutralized with saturated sodium bicarbonate solution until effervescence ceased. After further dilution with 10 ml. of water, the solution was extracted with three consecutive 20-ml. portions of chloroform, dried with magnesium sulfate, and evaporated under reduced pressure to dryness. Crystallisation occurred spontaneously. The material was recrystallised twice from methanol, washed with cold methanol, and dried at 50° for 3 hr.; yield 202 mg., m.p. 185-186°. Pure material was obtained on two more recrystallisations from methanol; m.p. 190-191°; [α]$_{D}^{24}$ +30° (c 1.53, chloroform), reported (53) m.p. 190-191°, and 33°. The x-ray powder diffraction data were identical with that of authentic materials: 11.19 w, 8.45 m, 6.56 w, 6.19 vs (1), 5.80 w, 5.31 s, 5.10 s, 6.27 m, 4.21 w, 4.08 w, 3.94 s, 3.77 wv, 3.65 w, 3.45 vs (2), 3.34 m, 3.10 s (3), 2.97 wv, 2.86 wv, 2.78 wv, 2.63 wv.

__Anal. Calcd. for C$_{23}$H$_{24}$N$_{2}$O$_{12}$: C, 53.08; H, 4.63; OCH$_{3}$, 17.9. Founds: C, 53.34; H, 5.01; OCH$_{3}$, 18.2."

The chromatographic evidence obtained together with that of the absolute identification of these two known derivatives leaves no doubt that the main repeating unit of the mannan of green coffee is (1 → 4)-β-D-mannopyranose (17).

C. **The Isolation and Characterisation of Green Coffee Cellulose**

1. **Nature of the starting material**

The holocellulose of green coffee is that material isolated after extensive extractions, reported earlier and herein briefly described
The ground green coffee beans were extracted successively at room temperature with ethanol:water, 8:2, benzene:ethanol, 2:1, water (3 times). The residual solids from the previous treatments were extracted twice with 0.5% ammonium oxalate solution at 90°. The holocellulose was prepared from this residue essentially according to the modification by Whistler and associates (57) of the procedure of Wise and co-workers (58) by treatment with aqueous, acidified sodium chlorite (pH 4.5 to 5.0) under nitrogen at 75°C for 15 hr.; yield 42% (dry basis). This material, after extraction with 10% potassium hydroxide, was previously (8,12,13) reported to contain the following amounts of constituent sugars:

- D-glucose, 17.8%;
- D-mannose, 48.5%;
- D-galactose, 14.8%;
- L-arabinose, 6.0%,

all as determined by acid hydrolysis, qualitative papergam densitometry, and derivatisation (13).

2. **Preparation of the formic acid insoluble holocellulose of green coffee**

An amount of 40 grams of this material (the holocellulose) was extracted for 4 days with hot formic acid (90%) in a Soxhlet extractor fitted with a coarse, sintered-glass funnel (20). The formic acid was removed from the residual holocellulose by evaporation under reduced conditions.


(58) L. E. Wise, M. Murphy, and A. A. D'Addieco, Paper Trade J., 122, 35 (1946).
pressure followed by the addition and removal of water by distillation under reduced pressure. A colorless solid was obtained on freeze-drying; yield 9.0 grams (22.5%), ash (sulfate) 1%.

3. **Hydrolysis of the formic acid residue**

The solvation and hydrolysis of this fraction was brought about by a similar procedure as that described earlier (12,13), and in this dissertation. In that section concerned with the hydrolysis of the (10% potassium hydroxide)-insoluble holocellulose of green coffee (holocellulose A) solvation was effected using absolute sulfuric acid. The solvation was more complete in a much shorter time when the formic acid insoluble holocellulose was dissolved in sulfuric acid adjusted to the fuming side (m.p. -8 to -10°C).

A 0.500-gram sample of the formic acid residue was added to 75 ml. of sulfuric acid (melting point -8 to -10°C) in a round-bottomed flask submerged in a sodium chloride-ice water bath at -4°C. (13). The flask was allowed to warm to 6°C over a period of 8 hr. when the amber-colored solution was essentially homogeneous. Under power stirring, the solution was frozen about the flask walls. Water was added slowly (while stirring) to the frozen material over several hours with continued cooling of the flask. The homogeneous solution was diluted to 3 liters giving a sulfuric acid concentration of approximately 3%. This solution was refluxed for 10 hr. and neutralized with barium carbonate. The solids were removed by filtration and the combined filtrate and washings were evaporated under reduced pressure to 1 liter. The process of filtration and concentration was repeated several times. The hydrolysate was taken to a sirup
under reduced pressure, refluxed with 100 ml. of methanol for 1 hr., and filtered. The combined filtrate and methanol washings were evaporated under reduced pressure to a sirup, which was dissolved in water, filtered through hardened filter paper, and diluted to 50 ml.

4. Assay of glucose in the formic acid residue

The sirup obtained above was chromatographed and quantitatively evaluated according to procedures described earlier (12,13).

Quantities of this solution ranging from 0.010 to 0.075 ml. were applied from a microburet to paper chromatograms, and the sugars present were obtained by development with ethyl acetate, acetic acid, and water (9:2:1), and indication with aniline phthalate reagent (47). The sugars present were determined densitometrically, yielding: glucose, 39% (basis theoretical hydrolysis yield); mannose, trace.

On another chromatogram a periodate-permanganate spray indicator revealed a spot with $R_f$ twice that of glucose which has not been further investigated.

5. The isolation of α-cellobiose octaacetate from the formic acid residue

The procedure of Green (59) was modified (20). An amount of

(59) J. W. Green, in "Methods in Carbohydrate Chemistry,"

2.00 grams (6.5% moisture) of the above-described formic acid residue was kneaded into a mixture of acetic anhydride (8 ml.) and sulfuric
acid (0.2 ml.), at ice-bath temperature, until completely wetted. The mixture was placed in an oven at 50°C for 14 days. The resulting black solid was mixed with acetic acid (10 ml.) until the solids were suspended. The suspension was stirred with 500 ml. of cold water and transferred to a larger container with an additional 250 ml. of water. The whole was then stirred for 30 minutes. The solids were removed by filtration, washed free of acid with water, air-dried, and extracted with boiling ethanol (95%, 175 ml.). Crystals were obtained on cooling; yield 0.290 grams, melting point 220-221°C. Concentration of the mother liquor gave additional material; 0.095 gram, melting point 220°C. The above aqueous filtrate was extracted with chloroform (200 ml.). The chloroform extract was shaken with an aqueous solution of sodium bicarbonate and evaporated under reduced pressure to yield a solid. The ethanol-extracted residue was then extracted with chloroform (200 ml.) and the chloroform was removed to again yield a solid. The chloroform-extracted solids were combined and upon solvation in hot ethanol (95%, 75 ml.) produced crystals on cooling; yield 0.600 gram, melting point 220°C. Mother liquor concentration afforded an additional amount; 0.105 gram, melting point 217°C. The total combined crystalline material (1.09 grams, 26.5%) was recrystallized from ethanol (95%); yield 0.940 gram, melting point 223°C, unchanged on further crystallization or on admixture with authentic α-celllobiose octaacetate, [α]_D ^{22} 440° (g 3.0, chloroform), x-ray powder diffraction pattern identical with an authentic specimen (60).
(a) Control experiment with cotton linters

The above-described modifications were applied to the isolation of α-cellobiase octaacetate from cotton linters in an attempt to raise the 42% (highest) yield reported by Green (59). By following the procedure, employing the chloroform extraction, a yield of 48.5% of α-cellobiase octaacetate was obtained from cotton linters in the first and only attempt.

6. The isolation of a high glucose content residue from the formic acid insoluble holocellulose

The formic acid residue (obtained above) (10.0 grams) was stirred for 20 hours at 80°C., under nitrogen, with 30% potassium hydroxide (2.0 liters). The solids were removed by filtration, washed free of base, and dried; yield 5.40 grams. The (30% potassium hydroxide)-insoluble material (hereinafter called holocellulose B) was hydrolyzed with sulfuric acid (m.p. -8 to -10°) in the same manner as previously described and was found to contain 82% glucose, and no other sugar. This fraction represents 5.1% of the dry weight of the green coffee bean. This material was subjected to acetylation and failed to yield more α-cellobiase octaacetate (26.5%) than was earlier recorded.

(a) Cuprammonium hydroxide solubility

Cuprammonium hydroxide was prepared according to the method of Launer and Wilson (61). To cooled solution of sodium hydroxide was
added solid cupric chloride and stirred for 30 min. The cupric hydroxide was isolated by filtration (accompanied by cooling) and washed heavily. The wet solid was added to 29% cold ammonium hydroxide with stirring. After settling overnight the clear blue supernatant was decanted and filtered. To 20 ml. of the clear cuprammonium hydroxide was added cleaned cooper wire, and 0.093 gram of holocellulose B. The stoppered mixture was shaken briefly. After 15 minutes all of the material had dissolved and it was recoverable readily upon neutralisation with hydrochloric acid.

When this experiment was repeated on the above-described formic acid residue (which had not been treated with 30% potassium hydroxide), and with intermittent shaking for 1 day, complete solvation was not obtained.

7. **Determination of cellulose resistivity to formic acid hydrolysis**

In order to determine how much cellulose could have been lost in the process of isolating the high glucose content residue, a series of experiments were conducted using cotton linters.

The cotton linters (moisture content 1.7%) were transferred to a previously cleaned (hot cleaning solution), dried (constant weight) coarse sintered glass funnel. Weights were determined in all cases to the nearest milligram, and the starting sample weights ranged
5 and 10 grams. A Soxhlet extractor was employed and the time was recorded from when the sample first became thoroughly wetted with the hot 90.6% formic acid. Lengths of extraction time were 1, 2, 3, and 4 days. After the extraction was complete the formic acid was removed by water washings and then washed with 100 ml. of very hot 1.5% sulfuric acid. This latter action was deemed necessary after a carbonyl peak corresponding to formate ester was found in the infrared following the extraction. This peak disappears after the sulfuric acid wash. The residual cellulose (retained in the funnel) was then washed free of sulfuric acid with water and dried to constant weight at 80°, generally requiring 24 hours. The residual samples were checked for dryness by raising the temperature after the 80° treatment.

Duplicate samples were run for each time length and the averaged results are plotted in Figure 6.

D. The Isolation and Characterisation of the Arabinogalactan of Green Coffee

1. Introduction

The assumption shall be made that although the relative ratio of L-arabinose to D-galactose may vary somewhat in the arabinogalactan preparations, it is one and the same substance.

Wolfson and Laver (12) first isolated an arabinogalactan rich fraction in the mother liquor from the 18% sodium hydroxide extraction of the (10% potassium hydroxide)-holocellulose of green coffee (holocellulose A) while isolating the mannan (17). This material was hydrolyzed by the "formic acid method" by Wolfson and Laver (12).
Fig. 6.—The Determination of Cellulose Resistivity to Formic Acid Hydrolysis.
The paper chromatograms indicated L-arabinose (19.0%) and D-galactose (63.9%). The fraction represents about 2.8% of the dry weight of the green bean and is relatively inaccessible.

(a) Reinvestigation of the arabinogalactan rich fraction isolated by Wolf and Laver

A portion (0.162 g.) of the arabinogalactan fraction isolated by Wolf and Laver (mother liquor from the mannan isolation) was hydrolyzed with formic acid, and processed as described previously. This hydrolysate was loaded on paper chromatograms and developed with ethyl acetate, acetic acid, and water (9:2:2 v/v/v), and sprayed with aniline phthalate reagent. A heavily loaded paper treated in this manner produced in addition to the expected spots for galactose and arabinose, three previously undetected sugars. One of these three sugars could be tentatively identified as xylose. The fastest moving spot, probably a deoxyhexose, was not rhamnose. Of the three previously undetected sugars, xylose was present in about equal quantity to that of arabinose, and the other two sugars were present in only trace amounts. The following acetylation is an attempt to remove these trace sugars.

In the manner of Larson and Smith (62), 0.351 g. of this fraction

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was dispersed in formamide (50 ml.) which solubilized very nicely. To this was added pyridine (5 ml.) and acetic anhydride (5 ml.)
dropwise, with stirring in an ice-bath, over the period of 1 hour. The reaction mixture was allowed to come to room temperature and stirring was maintained for 24 hr. at which time the color of the solution had turned quite dark. The reaction mixture was then poured slowly (with stirring) into 500 ml. of ice and water. Immediately a white flocculent precipitate appeared and the flask was refrigerated overnight. The precipitate was gelatinous and could not be filtered. The filter paper and slurry were covered with acetone (200 ml.), solubilizing the slurry. The acetone was removed under reduced pressure causing reprecipitation of the white fibrous material. A slurry of water and the acetylated fraction was freeze-dried and yielded only a very small amount of a yellow glass.

2. **Investigation of the arabinogalactan contained in the water-soluble fractions complexed with protein**

(a) **Nature of the starting material**

As previously reported (8,13) (Table 1) the first water extraction of the residue obtained from the green coffee bean (after defatting with 80% ethanol and 2:1 benzene-ethanol) when poured into absolute ethanol (70% ethanol final concentration) afforded a precipitate. This precipitate was found to be composed of a nitrogenous material along with arabinose and galactose. The elemental nitrogen analysis of this precipitate was found to be 10.75% indicating about 65-70% protein if it is the source of nitrogen in this precipitate.

A portion of this material (0.330 g.) was hydrolyzed by heating at steam temperature for 4 hr. with formic acid (100 ml.), followed
by the usual hydrolyzate processing. The concentrated hydrolyzate was loaded on paper chromatograms, developed with ethyl acetate, pyridine, and water 3.6/1/1.5 (63), and sprayed with aniline phthalate spray reagent. The zones indicated arabinose, galactose and glucose. The galactose:arabinose ratio was approximately 2:1 with only a trace of glucose. The yield could be visually estimated (by comparison with the known sugar concentrations of the same chromatogram) at 30%. Xylose was eliminated here by the nature of the development.

(b) Attempted removal of the protein by acetylation

A portion (10 g.) of the ethanol-insoluble precipitate from the first water extraction of the green coffee bean was dispersed in formamide (300 ml.) (62) by vigorous shaking. To this mixture was added pyridine (100 ml.) and acetic anhydride (90 ml.) dropwise while vigorously stirring and cooling in an ice-bath. After the acetic anhydride was all added (1.5 hr.), the solution turned black and was allowed to come to room temperature and stirred for 24 hr. The reaction was quenched in ice-water and extracted with chloroform repeatedly. The chloroform was washed four times with water followed by four aqueous cadmium chloride washings to precipitate the pyridine. The solids of these precipitations were removed by filtration and the chloroform was dried with magnesium sulfate which was likewise removed. The chloroform was removed under reduced pressure and a
water slurry freeze-dried. The material so obtained was a dark glass and minute in quantity.

(c) Attempted removal of the protein by precipitation techniques

In the manner developed by Sevag (64), 1.6 g. of the protein-

(64) M. G. Sevag, Biochem. Z., 273, 419 (1934).

polysaccharide material was shaken for 3 hr. with water (150 ml.), chloroform (50 ml.), and 1-pentanol. The solution was centrifuged and at the chloroform layer a white suspension appeared. The upper portion contained a greyish mucous-like material which tested positive to ninhydrin. This process was repeated several times, always removing more protein, but polysaccharide along with it. After four such processes the material left was only about 10-20% of the starting material and still was heavy in the protein component.

In the manner of Bell and Young (65), 2.3 g. of the protein-

(65) D. J. Bell and F. C. Young, Biochem. J., 28, 882 (1934).

polysaccharide material was dissolved in water (1 liter) to which trichloroacetic acid (40 g.) was added (in order to make a 4% trichloroacetic acid concentration), and the solution was refrigerated overnight. Much material had settled out in this time and this was removed by centrifugation and discarded. The centrifugate was collected and chilled again with no further precipitation after several hours. The centrifugate was then poured into 5 liters of cold ethanol and refrigerated. After 3 days precipitation had again
taken place, and the solids which were removed by centrifugation gave a positive soda-lime fusion test for nitrogen. The centrifugate was concentrated under reduced pressure (removing the ethanol), and refrigerated again. After standing overnight a small amount of solids could be precipitated again by pouring into cold ethanol. These solids gave a positive soda lime fusion test and the ethanol solution gave essentially nothing upon evaporation under reduced pressure. The same results as from the Sevag technique were thus obtained: the protein when precipitated removes the polysaccharide with it.

(d) Attempted removal of the protein by oxidation

In the same manner used by Wolfrean and Plunkett (8) for the preparation of the holocellulose of green coffee the protein-polysaccharide was oxidized. A portion (5 g.) of the material was strongly stirred with water (150 ml.) to which 2-octanol (10 ml.) had been added (to prevent foaming) until well solvated. To this water solution sodium chlorite (8 g.) was added over an 80 min. period. Acetic acid was added in order to hold the pH between 4.5-5.0. A steady stream of nitrogen was passed over the surface and carried off the excess chlorine gas. The reaction was held at 60-65° for 1.5 hr. in this manner and brought to pH 6.8 with 30% aqueous potassium hydroxide. The contents of the reaction flask were dialyzed against running water for 3 days, concentrated under reduced pressure, freeze-dried and yielded 4.4 g. of a beautifully white
fluffy material. This oxidised material exhibited a rotation of $\left[\alpha\right]_{D}^{20} = -48.3^\circ$ (c 0.75, formic acid) and contained 11.2% nitrogen.

(e) The partial removal of protein by hydrolytic enzymes

To 6 liters of water was added trishydroxymethylaminomethane (150 g) and sufficient hydrochloric acid to make the pH of the solution 8.5. To this solution was added chymotrypsin (150 mg.), and the protein-polysaccharide mixture (5 g.). By wrapping the flask with toweling and stirring it by magnetic stirrer the temperature was maintained at 35-40°C. for 10 days. The mixture was then concentrated under reduced pressure (1 liter), dialyzed against water for 7 days, concentrated again under reduced pressure, and lyophilised; yield 2.5 g.


This procedure will be further described.

(f) The removal of protein by column chromatography

Sufficient DEAE-cellulose (diethylaminoethylcellulose, Schleicher and Schuell Co., Keene, N. H.) was soaked in 1% sodium hydroxide to pack a column (by gravity) 7 cm. in diameter to a depth of 76 cm. The column was washed with water until neutral to litmus. The protein-polysaccharide mixture (2.5 g.) in a dilute water solution was placed on the column and eluted with water. The following fractions were collected; given in order: volume, weight (after concentration and lyophilization), and elemental nitrogen analysis; 6 liters, 285 mg., 0.81%; 10 liters, 60 mg., 1.43%; and 20 liters,
65 mg., 2.2%; total yield 410 mg. (54% return of the starting carbohydrate material). This procedure shall be described in greater detail in the following section.

3. **The isolation of an arabinogalactan complexed with protein and aromatic material by hot water extraction of the second ammonium oxalate residue**

(a) **Extraction and nature of the starting material**

The second ammonium oxalate residue is what remains after the defatting and deproteinizing steps, and which has not been given the chlorine dioxide treatment (8) (Table 1). This residue (57 g.) was refluxed with water (2.5 liters) and 2-octanol (50 ml.) for 1 hr. The solids were removed by filtration, washed with water, and the combined filtrate and washings were concentrated and lyophilized; yield 3.0 g.

**Anal. N, 5.15 (Dumas).**

The residue from the first extract was refluxed for 5 hr. (same conditions) and the extract treated as above; yield 1.5 g.

**Anal. N, 2.8 (Dumas).**

The third extraction (5 hr.) treatment yielded a product weighing 0.65 g.

**Anal. N, 3.0 (Dumas).**

(b) **The removal of protein by column chromatography**

Four columns 10 cm. in diameter were packed to a depth of 20 cm. with freshly treated DEAE-cellulose, and washed free of base. These columns were placed in a series where the first was higher than the second, and so on, and the eluate passed continuously in that order
until collected after passing through the fourth column. The hot water extract from 32 g. (equivalent to 1.7 g., 5.15% N) of the second ammonium oxalate residue was diluted to 5 gallons and passed through the series of columns as described above. The total eluate (13 gal.) was concentrated under reduced pressure and lyophilized; yield 530 mg.

Anal. N, 0.35 (Dumas).

Although this procedure worked quite well it involved the handling of a large volume of eluate in order to obtain any significant amount of starting material. The yield was poor and the nitrogen analysis was low as the products contained 20-40% ash.

(c) Removal of protein by enzyme hydrolysis and isolation of an arabinogalactan-aromatic complex (Product A)

A large amount (800 g.) of the second ammonium oxalate residue was refluxed with water (20 liters) and 2-octanol (200 ml.) for 1 hr. (1). The solids were removed by filtration through medium sintered glass (while hot) and washed with water (2 liters). The extract was divided equally and placed in 2 (12 liter) Florence flasks. To each flask was added tris(hydroxymethylamino)methane and sufficient hydrochloric acid to obtain a pH of 8.5. Chymotrypsin (300 mg.) and trypsin (235 mg.) were added to each flask and the temperature was maintained at 35-40° while stirring magnetically for 12 days. During the digestion flocculent material had separated out of solution, and was removed by filtration through coarse sintered glass. The filtrate was concentrated under reduced pressure to several liters and dialyzed against distilled water for 9 days, concentrated
under reduced pressure and freeze-dried; yield 19 g. During repeated attempts to freeze-dry this material the physical appearance changed from that of an amorphous solid to one with crystalline characteristics. This material (Product A) indeed yielded an x-ray powder diffraction pattern, showed free carbonyl absorption at 5.75 μ (KBr) and aromatic absorption at 260 and 275 μ (max. Me2SO).

**Anal. N, 0.17 by normal Dumas, N, 2.7 by Kjeldahl, ash content 1.0% (both direct and sulfate methods).**

The x-ray powder diffraction data were recorded: 5.49 s (1), 5.01 w, 3.29 vw, 3.15 vw, 2.74 m, 2.45 m (3), 2.23 m, 2.05 s (2).

(d) **The saponification of the arabinogalactan-aromatic complex**

(1) **Saponification number**

The polysaccharide-aromatic complex (Product A) (0.54 g.) was stirred magnetically with standardized aqueous sodium hydroxide (50 ml., 0.205 N) at room temperature for 2 hr., which caused good solvation. The solution was stirred an additional 2 hr. at 55-60° and an aliquot was removed and titrated with standard hydrochloric acid using phenolphthalein as the indicator. The amount of base consumed indicated a saponification number of 1.55 meq./g. Further heating at 55-60° failed to alter this value significantly. This material failed to show free carbonyl absorption.

(2) **Isolation of the arabinogalactan**

In much the same manner as the above experiment, the polysaccharide-aromatic complex (Product A) (9.85 g.) was stirred with aqueous sodium hydroxide (500 ml., 0.205 N) for 3 hr. at 25° and 3 hr. at 55-60°C.
The saponificate was brought to pH 7.5 with sulfuric acid and extracted successively with the following solvents: chloroform (400 ml.), ethyl acetate (600 ml.), and ether (600 ml.). The aqueous phase was then dialyzed against distilled water for 5 days and the 16 liters of dialyzate was collected; 12 liters of fresh water were then added for an additional 5 days and this was also collected. The non-dialyzables (3 liters) were concentrated under reduced pressure and freeze-dried; yield 6.21 g. (63% or 0.7% wt. dry bean).

**Anal.**  N, 1.8 (extended Dumas, using more vigorous conditions). Because of the nitrogen content, further dialysis was effected on a 2.0-g. portion (without collecting the dialyzate) against water for an additional 5 days, the non-dialyzables were concentrated under reduced pressure, and freeze-dried; yield 1.8 g., [α]$_{22}^D$ -25° (c 0.4, water). This material failed to show free carbonyl absorption. For analytical purposes a portion was dried at 78°, 0.5 mm. Hg, over phosphorus pentaoxide for 24 hr.

**Anal.**  N, 0.22 (extended Dumas) Calcd: C, 44.74; H, 6.18 (based on one pentose; two hexose residues) Found: (after correction for 3.7% ash): C, 45.5; H, 6.37.

(e) **Assay of constituent sugars**

(1) **Material isolated from small scale saponification**

The polysaccharide (179 mg.), as isolated from a small scale saponification (saponification no.) was heated on a steam bath with 90% formic acid (100 ml.) for 2 hr. The formic acid was removed by evaporation under reduced pressure and successive codistillations with water. The hydrolysate was concentrated to a clear amber sirup
and refluxed with dilute sulfuric acid (30 ml., 1.5%) for 1.5 hr., cooled, neutralized with barium hydroxide, and the solids removed by filtration through medium sintered glass. The filtrate and washings were concentrated under reduced pressure, filtered and concentrated again. The resulting sirup was refluxed with methanol (100 ml.) for 1 hr. and filtered. The filtrate and washings were concentrated to a sirup and diluted to 10 ml. with water.

Quantities of the hydrolyzate ranging from 0.0060 ml. to 0.0300 ml. were applied to paper chromatograms along with a known solution containing arabinose and galactose and were developed according to the procedure of Quick (47). The chromatograms were air-dried, sprayed with aniline phthalate reagent, and heated in an oven at 100-115° for 15 min. The densities of the resulting spots were read as quickly as possible by a densitometer (Photovolt Electronic Densitometer, Model 5W, Photovolt Corp., New York, N. Y.) following closely the procedure of McFarren, Brand, and Rutkowski (48), as used by Wolfrom and Laver (12,13) on the (10% potassium hydroxide)-insoluble holo-cellulose of green coffee hydrolyzate. The results obtained gave a 33% yield of arabinose, and 62% yield of galactose. Total yield of sugars 95%, ratio 1:1.9. Up to this point the work on the arabinogalactan isolated from the second ammonium oxalate residue had been presented in a preliminary report (66).

(2) Material isolated from large scale saponification

When the same procedure using formic acid hydrolysis was employed on the material isolated from the large scale saponification (isolation of the arabinogalactan) a value of 58% was obtained for galactose and 18% for arabinose. The arabinose content was obtained by following the 2-furaldehyde method and a pentose value of 26% was obtained. This fraction then can be accounted for as 26% arabinose and 58% galactose, total yield 84%, ratio 1:2.2. Further attempts to approach the 95% yield recorded earlier did not meet with success.

(f) Methylation of the arabinogalactan

The arabinogalactan (2.0 g.) was stirred with dimethyl sulfoxide (200 ml.) (67) at 35-40° for 2 hr. and solvated quite well. To this was added dimethyl formamide (50 ml.) and the solution was cooled to ice-bath temperature. Barium oxide (17 g.), barium hydroxide octahydrate (17 g.), and dimethyl sulfate (40 ml.) were added. The reaction was stirred magnetically (while under an atmosphere of nitrogen) and after 5 hr. was allowed to come to room temperature. After 18 hr., additional reagents were added (8 g. of each base) and dimethyl sulfate (15 ml.), and again the same amounts 8 hr. later. After 48 hr. of stirring, the reaction was diluted with chloroform (2 liters), chilled, and the solids removed by filtration through 2 layers of glass filter paper. The filtrate and washings were evaporated under reduced pressure to 300 ml., chilled, and refiltered. The chloroform was removed,
and the slurry dialyzed (to remove the solvents) against water for 2 days, concentrated and codistilled with toluene to dryness, extracted with chloroform, and filtered. The chloroform was removed and a water slurry was freeze-dried; yield 0.75 g.

**Anal.** Calcd. for C_{17}H_{20}O_{6}(OCH_{3})_{8}: OCH_{3}, 43.4. Found (68): 23.3


The product from the first methylation was stirred with tetrahydrofuran (51) (250 ml.) and solvated quite well. The flask was chilled to ice-bath temperature, powdered sodium hydroxide (20 g.) and dimethyl sulfate (24 ml.) added, and after 3 hr. the mixture was allowed to warm to room temperature. An atmosphere of nitrogen was kept over the reaction. Additional reagents (one-half the above quantities) were added 3 times over the 72 hr. total reaction time. The reaction mixture was dialyzed against distilled water for 6 days, concentrated and freeze-dried; yield 650 mg.

**Anal.** OCH_{3}, 33.2%.

The twice methylated product was remethylated again in the same manner as the second. The freeze-dried product was extracted with chloroform, filtered through glass paper, and the filtrate and washings concentrated to dryness, showing no visible salts. The dry solid was again dissolved in chloroform (75 ml.) and added to chilled petroleum ether (250 ml., b.p. 30-60°) which caused a flocculent white precipitate to form. This was isolated (after several days of refrigeration) by decanting the solvents and slurring the solids with water followed by freeze-drying; yield 350 mg. **Anal.** OCH_{3}, 29.6%.
The thrice methylated material was dissolved in dimethyl sulfoxide (80 ml.), dimethyl formamide (60 ml.), cooled to ice-bath temperature and stirred magnetically (while under an atmosphere of nitrogen). To the solvated polysaccharide was added barium oxide (4 g.), barium hydroxide octahydrate (2 g.), and methyl iodide (6 ml.) (68). After 2 hr., the reaction was warmed to room temperature and stirred for a total of 67 hr. Chloroform (500 ml.) was added to the reaction mixture which was then chilled and the solids were removed by filtration through glass paper. The filtrate and washings were evaporated under reduced pressure (0.5 mm. Hg) to dryness, chloroform added (300 ml.) and the solids again removed by filtration. The chloroform filtrate was poured into chilled petroleum ether (500 ml., b.p. 30-60°) and refrigerated for several days. Material settled out which could not all have been organic. The solvents were removed by evaporation and a water slurry was dialyzed for 3 days against water. The non-dialyzables were evaporated to dryness, taken up in chloroform, filtered, evaporated to dryness to yield a yellow sirup apparently salt-free. A water slurry was made and freeze-dried; yield 300 mg.

Anal. CMe, 31.6%, ash 1.0%.

This material shows the free hydroxyl (3.0 KBr) almost completely absent. Further examination of the infrared spectra (KBr) revealed in addition to the expected absorption (corresponding to hydroxyl and C-H stretching) there were sharp peaks at the carbonyl wavelengths 5.75 and 6.1. The 5.75 again corresponds to an ester and 6.1 could be an amide. It appears possible that through the methylation procedure the foreign material (which was not removed by the saponification treatment) was
concentrated. Unfortunately an ultraviolet spectra was not taken.

(1) Chromatographic investigation

The product of the fourth methylation (300 mg.) was refluxed for 45 hr. with 4% methanolic hydrogen chloride (45), neutralized with silver carbonate, and filtered. The combined filtrate and washings were evaporated under reduced pressure to 10 ml., chilled, and filtered again. This clear amber solution was further concentrated to 5 ml. by air stream.

To obtain an estimate of the sugars contained in the above sirup the methyl glycosides were analyzed by gas-liquid partition chromatography (69). The column employed was prepared by dissolving Apieson M vacuum grease in chloroform and depositing the solution upon Celite (80 mesh retained) by evaporating the chloroform (20% liquid to solid phase). The column was 1/4 inch by 6 ft. copper tubing. The chromatograph used was the Beckman GC-2A. A trial mixture containing methyl-2,3,6-tri-$\alpha$-methyl-$\beta$-D-glucos-, and galactopyranosides in addition to methyltetra-$\alpha$-methyl-$\alpha$-D-mannopyranoside, was injected into the instrument by means of a Beckman microsyringe. The column temperature was $190^\circ$ and 70 ml./min. helium gas pressure was used. The three sugars separated nicely under these conditions. Upon application of the above sirup under the same condition three peaks were observed, retention times: 3.5, 7, and 9 minutes.

The sirup was also applied to thin layer plates made up with Silica
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Gel 0. (250 microns) and developed with benzene:methanol (93:7), air dried, sprayed with concentrated sulfuric acid, and heated for 15 min. in an oven at 160° (70,71). The eight zones so obtained had the


following \( R_f \) values and relative intensity, estimated visually: v, very; h, heavy; m, medium; l, light. 0.13 v.h., 0.18 l., 0.21 v.l., 0.26 h., 0.41 l., 0.43 l., 0.66 m., 0.90 h. The sirup was then loaded on thicker plates (500 microns) and the four heaviest zones were excised and eluted with methanol. The sirups were filtered, concentrated under reduced pressure and filtered again. The thin pale yellow solutions were then concentrated by air stream and refrigerated for several months. All attempts at crystallization failed.

4. Investigation of the arabinogalactan isolated from oxidized precursors

(a) Nature of the starting materials

As reported earlier (8,13) (Table 1) the second ammonium oxalate residue is treated with acidified sodium chlorite to produce the holo-cellulose of green coffee. This residue was then treated with 10% potassium hydroxide (under nitrogen) and the insoluble portion was called “holocellulose A.” Further treatment of this residue with 18% sodium hydroxide yielded the mannain (17) and an impure arabinogalactan. The extraction of these three materials to yield the
arabinogalactan in varying states of purity is herein described.

(b) The isolation of the arabinogalactan by extraction of the (18% sodium hydroxide)-insoluble holo-cellulose with dimethyl sulfoxide and formamide

A portion (20 g.) of the (18% sodium hydroxide)-insoluble holo-cellulose (Table 1) was vigorously shaken for 3 days with dimethyl sulfoxide (250 ml.). The solids were removed by filtration, washed with acetone, and dried at 60° for 3 days; yield 19.2 g. The acetone was removed from the solution under reduced pressure. Precipitation attempts were made with methanol, chilled Skellysolve B, and chilled ethanol, all to no avail. The dimethyl sulfoxide was removed by dialysis against water for 3 days. White flocculent material had separated out and the excess water was removed under reduced pressure. A slurry was made of the evaporated dialyzate and freeze-dried. The freeze-dried product weighed 440 mg. (a mechanical loss of about 20% was suffered during dialysis). A second dimethyl sulfoxide extraction of the once extracted material in the same manner throughout yielded upon freeze-drying an additional 100 mg. of a white fluffy powder.

A portion of the above product (80 mg.) was hydrolyzed by heating with formic acid (100 ml.) on the steam bath for 3 hr., followed by the usual hydrolyzate processing. The concentrated hydrolysate was loaded on paper chromatograms, developed with ethyl acetate, acetic acid, and water (9:2:2 v./v./v.) and upon spraying with aniline phthalate spray reagent showed only spots corresponding to arabinose and galactose despite intense loading of the sirup. The ratio of galactose to arabinose was approximately 2:1.
Another portion (8.5 g.) of the (18% sodium hydroxide)-insoluble holocellulose of green coffee was shaken vigorously for 5 days with formamide (300 ml.). The solids were removed by filtration, washed with formamide and the solution dialysed against water for 3 days, concentrated under reduced pressure and freeze-dried, yielding 98 mg. of a white fluffy solid. Hydrolysis of the material was not attempted. The yields from the above extractions were considered too low to be of isolative value.

(c) Isolation of an arabinogalactan by dimethyl sulfoxide extraction of (10% potassium hydroxide)-insoluble holocellulose (Holocellulose A)

Holocellulose A (100 g.) was vigorously stirred with dimethyl sulfoxide (1.5 liters) for 24 hr. The solids were removed by filtration through medium sintered glass. The extract was dialysed against tap water for 5 days, and distilled water for 2 days. The dialyzed liquid was concentrated under reduced pressure and lyophilized; yield 1.3 g. The same process was repeated on the once extracted solids and yielded another 0.85 g. for a total of 2.15 g.

(1) Estimation of L-arabinose

The method developed by Wolfrom and Plunkett (8,13) was used for this hydrolysis with some modifications. A 0.328 g. sample of the previously described arabinogalactan was added to 200 ml. of boiling 1.5% sulfuric acid and refluxed for 20 min., cooled, neutralized with barium carbonate and the solids removed by filtration. This was followed by two more filtrations (after concentration under reduced pressure). The combined filtrate and washings were concentrated
(under reduced pressure) to a sirup and refluxed with methanol (200 ml.). The solids were removed by filtration, the methanol removed under reduced pressure, and the hydrolyzate diluted to 25 ml. volumetrically with water.

Quantities of the hydrolyzate ranging from 0.0016 ml. to 0.0064 ml. were applied to paper chromatograms along with a known solution of arabinose and were developed according to the procedure of Quick (47). The results obtained gave a 10% yield of arabinose. Because of this low yield this means of isolation was also given up.

(d) Isolation of the arabinogalactan by hot water extraction of (10% potassium hydroxide)-insoluble holocellulose (holocellulose A)

Holocellulose A (5 g.) was refluxed with water (600 ml.) and 2-octanol (20 ml.) for 1 hr. The solids were removed by filtration through medium sintered glass. The filtrate was concentrated under reduced pressure and lyophilized; yield 300 mg. The above procedure was repeated on the once extracted solids (6 hr. reflux) and yielded an additional 180 mg. for a total of 480 mg.

(1) Hydrolysis and estimation of sugars

The arabinogalactan (first hot water extract, 0.5 g.) was refluxed with formic acid for 6 hr. The formic acid was removed under reduced pressure and the residue was refluxed with 0.5 M sulfuric acid to remove formate esters. The hydrolyzate was neutralized with aqueous barium carbonate and the inorganic components were removed as described earlier for the hydrolysis of the dimethyl sulfoxide extract. The sirup was diluted volumetrically with water to 25 ml. Quantities
of the hydrolysate ranging from .004 ml. to .016 ml. were applied
to paper chromatograms along with a known solution containing the
constituent sugars and were quantitatively estimated by the earlier
described method and a yield of 19% arabinose along with 25% galac-
tose was obtained.

(e) Isolation of the arabinogalactan by hot water
extraction of the holocellulose

The holocellulose of green coffee (Table 1) (500 g.) was stirred
with warm water (45 liters) and 2-octanol (100 ml.) overnight which
cased thorough wetting of the material. It was then refluxed (5 hr.)
and filtered through medium sintered glass and washed with water.
The combined filtrate and washings were evaporated under reduced
pressure to a dark brown solution (3 liters). The solution was made
basic (0.2 M NaOH) and it was stirred at 60° for 4 hr. under a strong
stream of nitrogen which caused it to further darken. The solution
was made neutral to litmus with sulfuric acid. Upon neutralization
the color again turned lighter. The neutralized solution was dialyzed
for 4 days against distilled water and the colored clear solution was
evaporated under reduced pressure to 1.5 liters and poured into cold
95% ethanol (final ethanol concentration, 70%). After standing for
2 days no flocculation had occurred although the mixture was very
turbid. Upon addition of a few grains of sodium chloride immediate
flocculation occurred. The solids were isolated by repeated decanta-
tion and 70% ethanol washings. The solids were slurried with water
and lyophilised; yield 11.4 g. of a light colored, fluffy solid
[α]₂⁸D = -28° (c 0.49, water). This represents a yield of 2.2% from
the holocellulose or 0.92% of the green coffee bean (dry weight basis). This material failed to show free carbonyl absorption. For analytical purposes a portion was dried at 78°, 0.2 mm. Hg, over magnesium perchlorate for 24 hr.

Anal. Calcd: C, 44.66; H, 6.12 (based on one pentose; three hexose residues). Found: C, 43.10; H, 6.65. Upon further drying the sample continued to lose water. Based on the above figures with 3% correction for water; Found: C, 44.79; H, 6.45.

The combined decantate and washings from the above precipitation was concentrated under reduced pressure and lyophilized; yield 6.0 g. of a grey-brown flakey solid.

(1) Hydrolysis and estimation of sugars

The arabinogalactan (630 mg.) was heated at steam bath temperature with formic acid (100 ml.) for 4 hr. The formic acid was removed under reduced pressure and the resulting sirup was heated at steam bath temperature with dilute sulfuric acid (50 ml., 1.5%) for 1 hr. The solution was neutralized with barium hydroxide and processed in the usual manner. The salt-free hydrolyzate sirup was diluted to 25 ml.

The quantitization of constituent sugars followed the procedure of Wilson (72). Quantities of the sirup along with a known solution


of arabinose and galactose were applied by means of a microburette to Whatman No. 1 paper. The sugars were developed with 1-butanol,
pyridine, and water (6:4:3 v/v/v) in a glass tank. The developed papers were air-dried for 4 hr. and drawn through a trough containing aniline hydrogen phthalate reagent and again air-dried, followed by heating at 105° for 10 min. The individual zones were excised in rectangular areas as nearly equal as possible. The papers were then cut into smaller strips and (those from each zone) placed in test tubes. The eluting solution (4 ml., 0.7 N hydrochloric acid in 80% ethanol v/v/v) was added to the test tubes and shaken several times over an elution time of 1 hr. The absorbances of the solutions were determined at 390 μm for galactose and 360 μm for arabinose. The absorbances of the known zones are plotted vs. their concentrations and the hydrolysate concentrations are then read directly from their absorbances. The results obtained gave a yield of 22.0% for arabinose and 61.5% for galactose. A known amount of arabinose and galactose were subjected to an identical hydrolysis and processing procedure and 96% of the arabinose was returned along with 94.1% of the galactose. Using these corrections the yield from the hydrolysate would be 24% for arabinose and 65% for galactose (average of 3 determinations).

This method (72) for determining the quantities of sugars in the hydrolysate is superior to that involving a densitometer as used previously. As shall be seen later, despite the 89% hydrolysis yield (±3%) the presence of foreign substances (other than carbohydrate) cannot be discounted. If said foreign substances are more resistant to repeated chemical manipulations they indeed can become concentrated to such an extent that the structural elucidation of the parent polysaccharide is vastly hindered.
5. The structural elucidation of the arabinogalactan from the hoolcelltulose of green coffee

a. The first partially successful series of methylations

Attempts were made to fully methylate the arabinogalactan using the Kuhn (67) method throughout. After four such attempts almost all the starting material was lost and the methoxyl content was not as high as obtained by the following procedure.

The polysaccharide (2.4 g.) was stirred at 40° with dimethyl sulfoxide (200 ml.) until well solvated. Dimethyl formamide (100 ml.) was added, the solution cooled, powdered sodium hydroxide (30 g.) and dimethyl sulfate (36 ml.) added under an atmosphere of nitrogen. The reaction was allowed to warm to room temperature after 4 hr. and stirred for a total of 24 hr. The reaction was then dialyzed and the non-dialyzables were extracted with chloroform (700 ml.), chilled, and filtered through Celite 545 suspended on medium sintered glass. The combined filtrate and chloroform washings (75 ml.) were poured into cold petroleum ether (300 ml.) and the solids which precipitated were slurried in 95% ethanol (the mother liquor was saved). The ethanol was gradually removed by evaporation with water, and the slurry was lyophilized; yield 0.8 g. of a light colored solid.

Anal. Calcd. for C_{23}H_{27}O_{9}(OCH_3)_{11}: OCH_3, 44.1. Found (68): 30.75.

The once-methylated, lyophilized material (760 mg.) was solvated in warm dimethyl sulfoxide (20 ml.) which was then cooled to 25°. To this solution was added barium oxide (12 g.) and methyl iodide
(10 ml.) (67). Unfortunately the reaction temperature rose rapidly after an induction time of several hours and a goodly amount of the reactants was lost by foaming. Additional solvent (40 ml.) was used to salvage the reflux condenser contents and the reaction was stirred for 24 hr. at approximately 30° and then dialyzed. The non-dialyzables were evaporated to dryness under reduced pressure. The solids were extracted with chloroform, chilled and filtered. The combined filtrate and chloroform washings were concentrated and filtered again. After final filtration the concentrated brown chloroform solution was poured into petroleum ether and the precipitated solids were isolated by decantation (dried overnight at 78° and 0.02 mm. Hg.).

Anal.: OMe, 38.12% (after correction for 5.6% ash). The hydroxyl absorption in the infrared was very well diminished (carbonyl absorption at 5.75 appeared). This reaction (using methyl iodide and barium oxide in dimethyl sulfoxide) was repeated on the precipitated product (38.12% OMe) and its mother liquor (not analyzed). The combined products from the second methylation (in this series) were remethylated by this same procedure except the reaction was kept under control. The material was dissolved in dimethyl sulfoxide (35 ml.) and methylated with barium oxide (12 g.) and methyl iodide (11 ml.). After several hours of cooling (below 25°) the reaction was contained, and stirred for 18 hr. at room temperature. At that time a similar amount of reagents were added (with cooling) and after the induction period time was passed the temperature was raised to 35-40° for 8 hr. and then stirred at room temperature overnight. To the reaction was added chloroform (300 ml.), the mixture chilled and
filtered through Celite 545. The chloroform was removed from the filtrate and washings by evaporation and the clear red colored solution dialyzed to remove the dimethyl sulfoxide. The non-dialyzables obtained on concentration to dryness were extracted with chloroform, concentrated (after filtration) and poured into cold petroleum ether. The precipitate was isolated by decantation; yield 79 mg.

**Anal. OCH$_3$, 32.89%.** Resinification took place in the reaction (analysis) vessel. The mother liquor was isolated as a sirup (dried to constant wt.); yield 86 mg. The hydroxyl absorption in the infrared was no stronger than earlier recorded (38.12% OCH$_3$) but the carbonyl absorption was enhanced.

The sirup (2.0 g.) (mother liquor) from the first methylation in this series (sodium hydroxide and dimethyl sulfate) was dissolved in warm dimethyl sulfoxide (50 ml.) and methylated with barium oxide and methyl iodide. After 24 hr. (35-45$^\circ$) black particles were floating on the surface. This observation was made by Kuhn (67) during an isolation of a fully methylated polysaccharide under these conditions. The reactants were dialyzed (without prior filtration). The non-dialyzables were concentrated to dryness and dissolved in chloroform (300 ml.), chilled, and filtered. The combined filtrate and washings (200 ml.) were evaporated under reduced pressure and the concentrated chloroform solution oiled out when poured into cold petroleum ether. The product was isolated as a sirup by drying in a vacuum oven for two days. A portion of the sirup was then poured into cold petroleum ether and the precipitate isolated by this procedure gave a good infrared spectrum from the standpoint of hydroxyl
absorption in relation to C-H stretching but showed carbonyl absorption at 5.8. The infrared suggested a methoxyl content of at least 30% but only 12.1% was obtained. Resinification again took place in the analysis vessel. The ultraviolet spectra showed "non-polysaccharide" absorption with maxima at 203, 205, 236, and a shoulder at 302-305 μ (95% ethanol). The n.m.r. of this material was not confirmatory of unsaturation as it showed only hydrocarbon character.

The remaining sirup (except for the 130 mg precipitated) was again methylated (3rd time) with barium oxide and methyl iodide in dimethyl sulfoxide, with additional reagents added twice over the 48 hr. reaction time (average temperature about 35°). The methyl iodide was driven off by heating the reactants diluted with chloroform (450 ml.), chilled, and filtered. The chloroform was removed by evaporation and the dimethyl sulfoxide by dialysis. The product was eventually isolated as a sirup one sixth of which was dried to a hard glass (180 mg.) which showed a very low methoxyl (8.62%). Again the infrared looked quite favorable (as good as material which had analyzed 38%) for methoxyl content but a carbonyl peak was observed at 5.8 μ. The methylation procedures which took place after the first one in this series (modified Haworth using dimethyl sulfoxide and dimethyl formamide) seemed on the whole only to isolate material which was more and more contaminated with "foreign" substances. These substances had most likely undergone concentration during the methylation process. The last described product was hereby further investigated instead of further methylated.
(1) Chromatographic investigation

The sirup (approx. 1.0 g.) described above (prior to this section) was refluxed with formic acid (100 ml.) for 4 hr. The formic acid was removed by distillation (and codistillation with water) and the resulting sirup was refluxed with 0.5% methanolic hydrogen chloride for 22 hr. The product was neutralized with Duolite A-4 ion exchange resin which was removed by filtration and washed heavily with methanol. The methanol solution was decolorized with carbon and the solution concentrated to a clear amber sirup (5 ml.) under reduced pressure at low temperature.

The sirup was chromatographed on Silica Gel G using benzene: methanol (93:7) (70:71) as the developer. The thin-layer plate was sprayed with sulfuric acid and heated. The five zones so obtained had the following $R_f$ value and relative intensity, estimated visually:

- v, very; h, heavy; m, medium; l, light; 0.073 h., 0.169 l., 0.245 h., 0.252 m., 0.338 h., with several faster moving, very faint zones.

Material which had moved out only slightly from the point of application showed luminescence under and ultraviolet lamp. The approximate relative intensity in terms of ratios would be: 5:1:3:2:3 in order of increasing $R_f$.

When this sirup was chromatographed alongside that resulting from the arabinogalactan after mild acid hydrolysis and then methylation, all the zones were found in that sirup with the exception of the last one herein noted at $R_f$ 0.338. Likewise all the zones in the latter sirup corresponded to those found in this sirup except for one pair.
(2) **Isolation of methyl 2,4-di-O-methyl-β-D-galactopyranoside**

The sirup from the above methanolysis was chromatographed alongside known methyl 2,4-di-O-methyl-β-D-galactoside and the slowest moving zone corresponded to that of the known sugar. Crystals formed in the sirup which were isolated by methanol washing and air drying: m.p. 153°. Accepted for methyl 2,4-di-O-methyl-β-D-galactopyranoside 166° (73).


(3) **Hydrolysis of the glycosides**

Zones corresponding to the slowest moving, Rf 0.073, 0.245 and 0.252 together, and 0.338 were isolated by preparative silica gel thin-layer chromatography, as earlier described. The three sirups so isolated were hydrolyzed with 1 N hydrochloric acid. The hydrogen chloride was removed by co-distillation with 2-propanol and chromatographed on "Avirin" cellulose plates employing butanol:ethanol:water 3:1:1 and indication with aniline phthlate. This method is to be described in detail in the next section. The slowest moving zone (after hydrolysis) corresponded to known 2,4-di-O-methyl-D-galactose. The next pair hydrolyzed to essentially one zone corresponding to authentic 2,4,6-tri-O-methyl-D-galactose. The faster pair hydrolyzed to essentially one zone also, which corresponded to 2,3,5-tri-O-methyl-L-arabinose.

b. The second series of methylations

The arabinogalactan (4.5 g.) was stirred for 24 hr. with dimethyl
sulfoxide (300 ml.) which caused good solvation. Dimethyl formamide (100 ml.) was added and the solution cooled and swept with nitrogen. Dimethyl sulfate (36 ml.) and powdered sodium hydroxide (36 g.) were then added and the reaction kept cold for 3 hr. Additional reagents (18 ml. of dimethyl sulfate and 15 g. of sodium hydroxide) were added after 22 hr. and 30 hr. After a total reaction time of 53 hr. the reaction was dialyzed. The non-dialyzables were concentrated and lyophilized; yield 5.3 g. of a hard brown solid. The material was remethylated in the same manner to yield approximately 5.0 g. of a hard brown solid. The infrared showed the hydroxyl pretty well covered and as yet no carbonyl absorption.

Anal. OCH₃ 30.49.

The solids were shaken with dry tetrahydrofuran (300 ml.) for 1 hr. and allowed to stand for 2 days. They were not very soluble in the tetrahydrofuran so that solvent was removed by co-distillation with dimethyl sulfoxide and a third methylation was carried out using solid sodium hydroxide and dimethyl sulfate. The twice methylated product dissolved very well in dimethyl sulfoxide (300 ml.) by stirring overnight. Dimethyl formamide (100 ml.) was added and the solution chilled and swept with nitrogen. Solid powdered sodium hydroxide (10 g.) and dimethyl sulfate (12 ml.) were added and the reaction kept cold for 3 hr. Additional equal amounts of the above reagents were added after 7, 26, 32, 48, and 56 hr. The reaction was dialyzed after a total reaction time of 5 days. The non-dialyzables were concentrated under reduced pressure to dryness, suspended in chloroform and filtered through Celite 545. The clear amber
filtrate and combined washings (1 liter) were concentrated to 10 ml. and poured into cold petroleum ether (200 ml.). A flocculent white solid precipitated which was isolated by centrifugation. The solid upon drying resulted in a horny, brown solid; yield 2.1 g.

Anal. OCH₂, 36.5% [α]²⁸D = -51° (c 0.56, chloroform). The infrared spectrum showed very diminished hydroxyl and sharp carbonyl absorption as in the previous series of methylations.

(1) Hydrolysis and chromatographic investigation using micro-crystalline cellulose as a thin-layer absorbent

The thrice-methylated product (0.835 g) was refluxed with formic acid (100 ml.) for 3.5 hr. The formic acid was removed by evaporation under reduced pressure and co-distillation with water. The resulting sirup was refluxed with dilute sulfuric acid (1.5%) for 3 hr., and neutralized with Duolite A-4 ion exchange resin.

The neutral concentrated sirup was paper chromatographed using butanol:ethanol:water 3:1:1 and indicated with aniline phthalate. Three main zones were observed (plus several very faint, slower moving zones) \( R_6 \), (g = 2,5,4,6-tetra-O-methyl-D-glucose) 1.07, 0.76, and 0.575. Evidently these are excellent separations. Thin-layer chromatography employing silica gel as an absorbent was quite unrewarding. Some separations were obtained but never enough to provide any remote possibility of using this technique for isolative purposes.

With the preparative or isolative aspect in mind it seemed that only heavy paper chromatography could be employed. While this technique is often successful (17), it is quite tedious. While cellulose thin-layer has been tried with limited success it seemed that perhaps
an unusual type of cellulose with inherent bood binding properties
might be the answer. This product proved to be "Avirin," a micro-
crystalline cellulose produced by American Viscose Company. "Avicel"
is the pharmaceutical grade of the same material. After much experi-
mentation this author determined that thin-layer plates prepared by
blending (Waring blender) 100 g. of the above product with 430 ml. water
for 15-45 sec. was an optimal slurry. The best thickness was 1.0 mm.
and the plates could either be air-dried overnight or in an oven at
80° until the surface moisture was lost (30-60 min.). Since this
discovery many conditions and types of separations have been tried
and in all cases this much faster than paper thin-layer method
affords at least equal and often better results than those obtained
with paper using the same solvent systems (74,75).

& Ind., 1065 (1964) (preliminary communication).

(75) M. L. Wolfrom, D. L. Patin, and R. M. de Lederkremer,
J. Chromatog., in press.

Further characterization of 2,4-di-O-methyl-D-galactose

(1) The isolation and characterization of 2,4-di-O-
methyl-D-galactose

The slower moving zone ($R_f$, 0.632) was isolated as a sirup by
preparative avirin cellulose thin-layer chromatography (74,75). The
free sugar was crystallized and it's hydrate from 95% ethanol, m.p.
74-8°, recrystallized 3 times to 93-5°, reported 103° (73). The
x-ray pattern diffraction data were identical with authentic specimen:
11.11 m, 9.25 s (1), 7.38 w, 5.86 m, 5.56 m, 4.97 m, 4.27 s (2), 4.17 w, 3.98 m, 3.62 s (3), 3.16 s, 3.01 m, 2.93 m, 2.75 w, 2.60 m, 2.52 w, 2.45 w, 2.30 w, 2.28 m, 2.24 w, 2.18 w, 2.10 w, 2.05 m.

(2) The preparation of 2,4-di-O-methyl-N-phenyl-D-galactosylamine

The crystalline mass obtained above (0.075 g.) was stirred at room temperature with sulfuric acid (2 ml., pH 4.0), ethanol (5 ml.), and freshly distilled aniline (1 ml.) for 15 min. The excess aniline was removed by co-distillation with ethanol and spontaneous crystallization occurred to yield 55 mg. of the expected amine, m.p. 195-200°, recrystallized to 210-11°, reported 216° (73).

d. The characterization of 2,4,6-tri-O-methyl-D-galactose as the N-phenylamine

The zone corresponding to known 2,4,6-tri-O-methyl-D-galactose was isolated as a sirup by preparative thin-layer chromatography as earlier described (74,75). The sugar (0.080 g.) was reacted in the same manner as previously described with acidic ethanolic aniline to form the N-phenylamine. After stirring for 15 min, crystals formed (49 mg.) m.p. 164-5°, raised to 168-9° upon recrystallization from 95% ethanol, reported 170° (76). X-ray powder diffraction pattern


identical with authentic specimen kindly furnished by Professor G. A. Adams: 13.73 m, 11.76 s (3), 9.75 m, 8.24 s (2), 6.93 s,
6.40 vw, 5.69 m, 5.16 w, 4.85 w, 4.38 m, 4.16 s (l).


The characterization of 2,3,5-tri-O-methyl-L-arabinose as the amide

An unsuccessful early attempt at this procedure dictated a larger amount of pure starting material. A total of 40 preparative cellulose plates were developed and only the upper part of the indicated zone (R$_g$ 1.07) was excised and eluted with methanol. Centrifugation followed by several concentrations and filtrations removed all of the cellulose. The sirup weighed 0.235 g. and ran identical with authentic 2,3,5-tri-O-methyl-L-arabinose.

The sirup was dissolved in water (6 ml.) and stirred with bromine (1 ml.) for 66 hr. at room temperature in the dark (77).


The excess bromine was removed by aeration, the solution diluted with water (50 ml.) and extracted thrice with 50-ml. portions of chloroform. The chloroform extract was concentrated to a brown sirup and dried by co-distillation with toluene. The dried sirup was dissolved in liquid ammonia (75 ml.) which almost completely evaporated overnight. The remaining ammonia was removed by distillation with chloroform. The resulting sirup was nucleated with an authentic specimen kindly furnished by Professor Smith and yielded crystals, 44 mg., m.p. 127-9°, recrystallized to 133-5°, reported 138° (77).

The crystals when dissolved in methanol and chromatographed on Silica
Gel G using benzene:methanol 90:10 showed one zone when indicated with sulfuric acid. The mother liquor showed three zones the most intense of which was the desired product. The slowest moving probably was unreacted lactone and the fastest was probably an oxidation by-product. The x-ray powder diffraction pattern was identical to that of an authentic specimen kindly furnished by Professor F. Smith:

$8.71 s (1), 7.67 w, 6.34 m, 6.00 s (2), 5.60 w, 5.01 s (3), 4.39 w, 4.10 s, 3.78 m, 3.61 m, 3.43 w, 3.25 w, 2.80 w, 2.74 w, 2.52 w,$

$[\alpha]_{D}^{28} +18^\circ$ (g 1.3, water), reported $+16^\circ$, water (77).

(f) **The determination of relative amounts of methylated sugars in the hydrolysate**

The sirup hydrolysate was developed on paper chromatograms using butanol:ethanol:water 3:1:1, as previously described (45) and the zones were indicated with aniline hydrogen phthalate. The sirup was loaded by means of a microburette and the relative intensities of the 2,4-di-O-methyl and 2,4,6-tri-O-methyl-D-galactose were read from five zones by means of the densitometer. The results indicated a relative ratio of 1:1.47 of di- to tri-O-methyl derivative. Comparing this to the earlier recorded ratio of D-galactose to L-arabinose of 2.7:1 for the polysaccharide one arrives at the following ratios for the three constituent sugars: 2,3,5-tri-O-methyl-L-arabinose, 2,4-di-O-methyl-D-galactose, and 2,4,6-tri-O-methyl-D-galactose, 5:5.5:8, respectively.

g. **Partial hydrolysis and subsequent methylation of the arabinogalactan**

The arabinogalactan (8.3 g.) was added to hot dilute sulfuric acid (500 ml., 2.5%) and brought to reflux. The solution was
refluxed for 20 min., cooled and dialyzed. The non-dialyzables
(neutral) were concentrated to 300 ml. and poured into 1.5 liters of
chilled ethanol. The solids were isolated by decantation and evapo-
rated under reduced pressure with dimethyl sulfoxide (300 ml.) to
remove the ethanol and water. The material was then methylated using
sodium hydroxide and dimethyl sulfate. The product of the first
methylation was isolated by dialysis and lyophilization; yield 1.75 g.

The infrared showed no absorption in the 5.8 μ region.

This material was remethylated in the same manner and again the
product was isolated by dialysis and lyophilization; yield 1.4 g.

**Anal. Calcd.** for C₆H₈O₂ (OCH₃)₂: (2/3 methylation):
OCH₃, 30.33; C, 50.51; H, 7.42. Found: OCH₃, 27.4; C, 52.25; H, 7.66.
Even though both reactions were run in the same manner and products iso-
lated identically, the product of the second methylation has a lower
methoxyl content than that of the first; yet the C-H analysis indicates
higher methylation. The infrared in terms of relative hydroxyl ab-
sorption to C-H stretching is without doubt vastly superior in the
second product. A shoulder is starting to appear at 5.8 μ.

The product isolated from the second methylation was remethylated
following the procedure of Kuhn (67). The partially methylated poly-
saccharide (1.04 g.) was dissolved in dimethyl sulfoxide (50 ml.) to
which barium oxide (10 g.) and methyl iodide (12 ml.) were added.
More methyl iodide was added (12 ml.) after 8 hr. and 24 hr. The tem-
perature was kept between 35-40° for most of the reaction time of 48
hr. The methyl iodide was driven off, chloroform (200 ml.) added and
the mixture chilled and filtered. The combined filtrate and washings were evaporated under reduced pressure and dialyzed. The non-dialyzables were concentrated to dryness (with toluene) and extracted with chloroform. The chloroform extract (50 ml.) was chilled, filtered, and concentrated to 5 ml. The concentrate was poured into cold petroleum ether (100 ml.). The white solids were isolated by decantation; yield 400 mg.

**Anal.** Calcd. for C₆H₁₇O₂ (OCH₃)₃ (full methylation): OCH₃, 45.5; C, 52.93; H, 7.99. Found: OCH₃, 35.2; C, 52.11; H, 5.53. The infrared absorption showed a small amount of hydroxyl and a sharp carbonyl peak at 5.8 μ. The three states of methylation in terms of infrared absorption are shown in Figure 7. This figure shows the picture of what has been taking place during all of these methylation studies.

(1) **Methanolysis and chromatographic investigation**

The above-described product (300 mg.) was refluxed with methanolic hydrogen chloride (4%) for 22 hr (45). The solution was neutralized with Duolite A-4 ion exchange resin which was removed by filtration. The filtrate and washings were concentrated under reduced pressure to yield an amber colored sirup which was chromatographed on Silica Gel G plates using 7% methanol in benzene. Six zones were indicated by sulfuric acid spraying and heating. \( R_f \) values and relative intensity, estimated visually; 0.073 h., 0.169 m., 0.190 h., 0.210 h., 0.392 h., 0.450 m. The approximate relative intensity in terms of ratios would be: 3:2:2:2:2:1 in order of increasing \( R_f \). The first and slowest moving zones were identical to those found in the methanolyzate of the
Fig. 7.—The Stages of Methylation by Infrared Absorption.
full polysaccharide (that which had not been prehydrolyzed) and are therefore common to both products. The fastest two moving zones were proven to be methyl-tetra-α-methyl-D-galactose. The sirup was loaded on 0.5 mm. plates and the zones indicated on the edges with sulfuric acid. The zones were removed by scraping, were then allowed to stand in methanol for 10 hr., filtered and concentrated several times to remove the absorbent.

(2) The characterization of 2,3,4,6-tetra-α-methyl-D-galactose as the N-phenylamline

The fastest moving pair of zones were isolated as described above by the use of Silica Gel G layers (0.5 mm.). The sirup was refluxed with normal hydrochloric acid for 24 hr. The acid was removed by co-distillation with propanol. The sirup was chromatographed on a cellulose thin-layer plate alongside authentic tetra-α-methyl-D-galactose. The main zone exhibited the same $R_f$ as the authentic material.

The sirup was reacted with aniline and processed as described earlier for the other two galactose derivatives. Upon evaporation of the solvent a small amount (11 mg.) of crystals were obtained: m.p. 193°, reported 192°, 197° (78,79), x-ray powder diffraction data


identical with authentic specimen: 8.54 s (1), 6.77 m, 6.40 w, 5.45 m, 5.16 w, 5.02 w, 4.56 s (3), 4.25 m, 4.04 s (2), 3.89 w, 3.77 m, 3.57 w, 3.46 vw, 3.29 w, 3.16 w, 3.08 w, 2.96 vw. Anal. Calcd. for $C_{16}H_{27}NO_6$: $\text{OCH}_3$, 37.7. Found: $\text{OCH}_3$, 37.6.
IV. DISCUSSION OF RESULTS

A. Hydrolysis and Quantitative Assay of the (10% Potassium Hydroxide)-Insoluble Holocellulose (Holocellulose A) of Roast Coffee

In accord with the goal in mind of establishing a more fundamental knowledge of the coffee bean, it seemed advisable to compare the constituent sugar content of holocellulose A in both the green and roast bean, the preparation of which is described in Table 1. This, as a first approximation, would lend knowledge to possible sugar alteration on roasting in this large carbohydrate reserve of the coffee bean. The hydrolysis was carried out by first dissolving holocellulose A in sulfuric acid adjusted to the fuming side, m.p. -8 to -10°. Although the earlier work (13) was with absolute sulfuric acid, better solvation was experienced with this method. The material was well in solution after 8 hr. and then (with cooling) was diluted to a 5% acid concentration. Refluxing was started and the degree of hydrolysis followed by the copper reduction method (46).

The quantitative estimation of sugars followed the densitometer method wherein paper chromatograms are loaded with known amounts of the standard saccharides. The hydrolyzate diluted to a known volume is added to the same chromatogram. This method is limited by two main factors. The most important of which is the care and patience taken with loading the chromatogram with the microburette. If the spots are kept the same size and a heat gun used in between the repeated applications, good reliability can be obtained with most sugars.
The use of the densitometer is also a limiting factor inherent with the instrument. The size of the aperture is regulated to insure the inclusion of the most intense area of the indicated zone.

Since this particular phase of the work has been completed we have turned to the method of Wilson (72) where the chromatograms are loaded and developed the same way but the indicated zones are excised and eluted with dilute ethanolic hydrochloric acid and the intensities of the solutions measured with the Beckman DU spectrophotometer. This method is superior due to the use of the whole zone and the instrument employed.

1. **Comparison of results from both the total hydrolysis of the (10 potassium hydroxide)-insoluble holocellulose of green and roast coffee (holocellulose A).**

Essentially the same procedure was followed in both determinations. As explained in the historical section, Wolfrom and Laver (12,13) investigated holocellulose A of green coffee isolated by the procedure outlined in Table 1. Holocellulose A of roast coffee was isolated in the same manner and hydrolyzed as described above by this author. The results of both investigations are described in Table 2.

As the table indicates, the results are quite close within experimental error. One would assume from this that no great change in the basic saccharide content of the hard polysaccharide core of the seed takes place upon roasting, at least as far as destruction of the sugars are concerned. There is, however, a possibility of linkage shifting as is evidenced when starch is subjected to roasting.
<table>
<thead>
<tr>
<th>Saccharide</th>
<th>Per cent saccharide</th>
<th>Green (12,13)</th>
<th>Roast</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-mannose</td>
<td></td>
<td>48.5</td>
<td>51.4</td>
</tr>
<tr>
<td>D-glucose</td>
<td></td>
<td>17.8</td>
<td>17.7</td>
</tr>
<tr>
<td>D-galactose</td>
<td></td>
<td>14.8</td>
<td>11.9</td>
</tr>
<tr>
<td>L-arabinose</td>
<td></td>
<td>6.0</td>
<td>6.7</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>87.1</strong></td>
<td><strong>87.6</strong></td>
</tr>
</tbody>
</table>
B. The Structural Elucidation of the Mannan of Green Coffee

As described in the historical section of this dissertation, Wolfrom and Laver (12,13) isolated the mannan of green coffee and started the structural elucidation. Laver carried it to the point of fully methylating the polysaccharide and tentatively identifying the main repeating unit to be that of 2,3,6-tri-O-methyl-D-mannopyranose by paper chromatography.

1. Preparation of known tri-O-methyl-D-mannopyranoses

The ivory nut mannan (Phytelephas macrocarpa) was methylated and hydrolyzed with formic acid to yield 2,3,6-tri-O-methyl-D-mannopyranose which was isolated from the lower methoxyl content products by preparative paper chromatography employing butanone-aacetone (52) as the developer. The syrup was reacted with p-nitrobenzoyl chloride (53) to yield the bis adduct, a known compound of good crystalline characteristics for purposes of comparison with our natural product.

The preparation (without isolation) of 2,3,4-tri-O-methyl mannopyranose is described but the saccharide was not needed so further discussion shall be avoided.

2. Hydrolysis and chromatographic investigation of the fully methylated mannan of green coffee

From a straight chain mannan with a homogeneous linkage one can expect four reasonably possible linkages in the pyranose configuration. The fully methylated mannan was hydrolyzed by the formic acid method and the tri-O-methyl-D-mannose was isolated by preparative paper
chromatography. The use of butanone-azeotrope (52) as a developer eliminated the possibility of a mixture of the possible saccharides except for the one resulting from a (1 → 2)-linkage (3,4,6-tri-O-methyl-D-mannose). With the (1 → 2) adjacent linkage open it is the only possible saccharide which would readily complex with borate ion. This sugar was therefore eliminated as a possible product of methylation by the failure of the isolated zone to complex with the borate buffer upon electrophoresis.

3. **Characterization of 2,3,6-tri-O-methyl-D-mannopyranose**

A small portion (100 g.) of the sirup isolated by preparative paper chromatography was converted in low yield to 2,3,6-tri-O-methyl-D-mannono-1,4-lactone (17 mg.) by oxidation with bromide water. The x-ray powder diffraction data yielded conclusive evidence that this was indeed the above product but a more satisfactory derivative was desired.

The sirup (150 mg.) was reacted with p-nitrobenzoyl chloride in pyridine at 60-70° for 30 min. Spontaneous crystallization occurred after the reaction mixture was processed by neutralization with sodium bicarbonate and extraction with chloroform (after removal of the solvent). The compound (202 mg., 84%) was isolated in good yield with excellent physical constants and the x-ray powder diffraction data were identical with that of an authentic specimen. The isolation of these two derivatives proved that the main repeating unit was (1 → 4)-D-mannopyranose and by the negative rotation one may assume the β-D-configuration (17).
This indicates that the mannan of green coffee possesses very much the same basic structure that most of the other common mannans found in nature. Their structures were all proven in much the same manner and their optical activities are quite similar as shown in Table 3 abstracted from reference (17). The molecular weight was determined by Dr. Robert K. Tubbs and Professor Quentin Van Winkle of this laboratory, to whom this author expresses his appreciation.

The described polysaccharide is one of the few mannans found in land plants; it constitutes 5% of the dry weight of the green coffee bean. Both the coffee bean and ivory nut are extremely hard seeds. In his review of mannans Aspinall (16) concludes that there are no essential differences in chemical structure between mannans A and B of the ivory nut and that they vary distinctly only in molecular size. The present author and Professor Wolfrom conclude, through a comparison of rotations and linkages (Table 3), that the only distinct difference between the mannans of the ivory nut, coffee bean, salep, seaweed, and probably other sources, is one of chain length. Indeed the occurrence of D-mannose linked β-D-(1 – 4) in these seeds and sea plants can be compared with the more common occurrence of cellulose in plants.

C. The Isolation and Characterization of Green Coffee Cellulose

The isolation of the holocellulose of green coffee is outlined in Table 1 (8,12,13). The saccharide content of this material after treatment with 10% potassium hydroxide had been determined by Wolfrom and Laver (12,13) and compiled along with that of the roast coffee bean
<table>
<thead>
<tr>
<th>Plant</th>
<th>Common name</th>
<th>Source</th>
<th>$[\alpha]_{15-20}^\circ$</th>
<th>$[\alpha]_{D}^{15-20}$</th>
<th>Degree of polymerization</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phytelaephas macrocarpa</td>
<td>Ivory nut mannan A</td>
<td>-28</td>
<td>-46</td>
<td>10-13</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ivory nut mannan B</td>
<td>-20</td>
<td>-</td>
<td>38-40</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>-26</td>
<td>-48</td>
<td>80</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Porphyra umbilicalis</td>
<td>Red alga (edible)</td>
<td>-22</td>
<td>-</td>
<td>-</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>Orchis and Bulophilia</td>
<td>Salep</td>
<td>-</td>
<td>-44</td>
<td>1340</td>
<td>83,84</td>
<td></td>
</tr>
<tr>
<td>Coffee arabica</td>
<td>Green coffee</td>
<td>-22</td>
<td>-</td>
<td>45</td>
<td>17</td>
<td></td>
</tr>
</tbody>
</table>

(84) F. Klages and R. Neumann, Ann., 523, 224 (1936).
in Table 2. To determine whether or not cellulose was indeed the glucan present (approx. 18% D-glucose) the problem was the usual one of separation from the other components.

1. **The preparation, hydrolysis, and acetylation of the formic acid insoluble holocellulose of green coffee**

The selective formic acid hydrolysis of the holocellulose of green coffee resulted in the isolation of a crude fraction which upon hydrolysis and quantitative assay revealed a D-glucose content of 39% with a trace of mannose. The initial hydrolysis was effected by nearly anhydrous sulfuric acid, a method described previously (13), except that better solution of the polysaccharide was obtained if the acid was adjusted, by its melting point, to slightly on the fuming (excess sulfur trioxide) side. This value for glucose is probably low as a good yield of α-cellobiose octaacetate was obtained upon acetylation of the residue following a modified procedure.

The procedure for cellulose acetylation, described by Green (59), was improved by adding a chloroform extraction as ethanol alone does not extract all of the product from the crude acetylyzate. Applied to cotton linters, a yield of 48.5% of α-cellobiose octaacetate was obtained as compared with the highest value of 42% reported by Green (59). Employing this improved method, a yield of 26.5% of α-cellobiose octaacetate was obtained from the crude cellulose preparation of green coffee.

2. **The isolation of a high glucose content residue from the formic acid insoluble holocellulose**

The treatment of the formic acid residue with hot 30% potassium hydroxide for 20 hours is to say the least a rigorous treatment
and resulted in a loss of 46% of the starting material. The product, however, when hydrolyzed and quantitatively assayed, gave a yield of 82% D-glucose (39% before base treatment). One may conclude that the previous treatment with hot formic acid followed by hot alkali had altered the polysaccharide so that it gave a lower yield of disaccharide, although affording a good yield (82%) of glucose upon hydrolysis. This material had good cuprammonia solubility (61) whereas the non-base treated did not.

All these criteria (82% glucose, α-cellulbiose octaacetate, and cuprammonia solubility) established the presence of cellulose in the green coffee bean. On the basis of the yields of α-cellulbiose octaacetate and glucose, the amount of cellulose present in the green coffee bean is 5.1% (dry basis). This work in no way alters the glucose content of the bean as previously reported (13) but establishes that at least 5.1% of the 7.5% glucose found is present as the β-D-(1→4)-glucan.

3. The determination of cellulose resistivity to formic acid hydrolysis

The results shown in Figure 6 indicate that cellulose is slowly degraded by hot formic acid. The degradation is indeed quite slow compared to that of the mannann and arabinogalactan of green coffee which provided the means for isolating cellulose. This author had hoped that this method might provide a means of getting the cellulose content of wood pulps with ease, but the degradation although slow is not slow enough for this purpose.
D. The Isolation and Characterization of the Arabinogalactan of Green Coffee

1. Reinvestigation of the arabinogalactan rich fraction isolated by Wolf from and Laver

Wolf from and Laver (12) first isolated an arabinogalactan rich fraction in the mother liquor from the 18% sodium hydroxide extraction of the (10% potassium hydroxide)-insoluble holocellulose which afforded the mannan (17). The hydrolysis of this fraction with formic acid and subsequent chromatographic investigation determined the presence of contaminating sugars. A significant amount of what is probably xylose was present in this small fraction (1.5% of the coffee bean) and two other unknown sugars. When the attempted purification by acetylation failed, the author and his adviser felt that a non-mother liquor source of the polysaccharide would provide a better chance of establishing the good criteria of purity necessary in a structure proof.

2. Investigation of the arabinogalactan contained in the water soluble fractions complexed with protein

As previously reported (8,13) (Table 1) the first water extraction of the residue obtained from the green coffee bean (after defatting with 80% ethanol and 2:1 benzene:ethanol) when poured into absolute ethanol affords a precipitate. This precipitate is largely protein but what carbohydrate is present appeared to be largely arabinogalactan.

Attempts were made to remove the protein by acetylation, precipitation techniques, oxidation, and hydrolytic enzymes. The enzyme treatment met with moderate success and was further refined and found useful as described in the next section. The use of column chromatography employing 2-(diethylaminoethyl)cellulose also was somewhat
successful, but this water-soluble source of the arabinogalactan was far too heavily complexed with protein to be useful in a structural investigation.

3. **The isolation of an arabinogalactan complexed with protein and aromatic material by hot water extraction of the second ammonium oxalate residue**

The second ammonium oxalate residue seemed to be a good starting point in these studies (Table 1). The defatting steps have been carried out without great difficulty and yet the material has not undergone any rigorous treatments. The use of refluxing water is a simple procedure which carries many advantages. If one wishes to isolate the extract as a solid, concentration and lyophilization are the only necessary steps. On the other hand, either of the two purification steps (for removal of protein) are conducted on aqueous solutions so that the extract may be used directly.

a. **The removal of protein by column chromatography**

This method involving the use of large columns packed with 2-(diethylaminoethyl)cellulose does successfully remove the complexed protein. The disadvantages are apparent however when one considers the large amount of eluent necessary, and its evaporation (25 gallons per isolated gram at best) in obtaining the product. The modified cellulose is also costly.

b. **The removal of protein by enzyme hydrolysis and isolation of an arabinogalactan-aromatic complex (Product A)**

This method was successful, and the experimental section contains the description of this run. The results obtained by using both
chymotrypsins trypsin 3:2 on the first hot water extract were the best obtained. Although considerable time is involved with one run, the system need not be attended. The isolation of a product from the reaction with a very low nitrogen content (0.17 normal Dumas and 2.7 by Kjeldahl) and in good yield in one operation seemed attractive. The higher Kjeldahl value might indicate the presence of aromatic nitrogen. Infrared and ultraviolet spectra of the enzyme-hydrolyzed first hot water extracted material from the second ammonium oxalate residue show a free carbonyl in the ester region and definite aromaticity, respectively. These data indicate quite definitely that we are dealing with a substance which is not completely carbohydrate in nature. Upon repeated attempts to freeze-dry the material a change in physical appearance took place and an x-ray powder diffraction pattern was obtained.

c. Isolation of the arabinogalactan and assay of constituent sugars

Upon saponification of the above described polysaccharide-aromatic complex (Product A) under mild conditions (50 parts of 0.205 N sodium hydroxide for 3 hr. at 25°, followed by 3 hr. at 55-60°), a polysaccharide was isolated (0.7% dry bean) which from all appearances was nearly pure. Mild acid hydrolysis (90% formic acid, 2 hr. steam bath temperature), followed by paper chromatography, and comparative densitometry revealed the presence of arabinose (26%) and galactose (58%). The polysaccharide gave good elemental analysis and showed optical activity [α]22D = -25° (c 0.4, water, 2-dm. tube).
d. **Methylation and chromatographic investigation**

The methylation procedures followed on this fraction were essentially those of Kuhn and Trischmann (67) employing dimethyl sulfoxide and \(\text{N}_2\text{H}_2\)-dimethylformamide with barium oxide, barium hydroxide (octahydrate) and methyl iodide or dimethyl sulfate. The theoretical value for full methylation is 43.4%. The first methylation resulted in 23.3% (68). The second raised it to 33.2% but with accompanying loss of material it dropped to 29.6% after three attempts and 31.6% after four. Perhaps the product was contaminated with foreign materials but the infrared shows almost no hydroxyl stretching and the appearance of two peaks in the carbonyl region at 5.75 and 6.1 μ.

The material was subjected to methanolysis and displayed three zones by gas-liquid chromatography and eight zones by thin-layer chromatography on silica gel. The four heaviest zones were isolated on 0.5 mm. Silica Gel G plates but failed to crystallize.

4. **Investigation of the arabinogalactan isolated from oxidized precursors**

Considering the above difficulty in fully methylating the polysaccharide when working with non-oxidized precursors, a new source was again considered. Several extraction methods all met with success but the most practical was that of using hot water on the holocellulose.

The holocellulose was refluxed with water for 5 hr. and concentrated somewhat. The solution was made basic and heated under nitrogen. The neutralized product was dialyzed, concentrated and poured into cold ethanol (70% final concentration). This follows a general procedure
for precipitating arabinogalactans and afforded a light colored solid upon lyophilization which analyzed quite well and failed to show free carbonyl absorption.

a. **Hydrolysis and estimation of sugars**

The polysaccharide was hydrolyzed in the usual manner with formic acid. The sugar content was estimated by the procedure of Wilson (72) employing an elution technique of the indicated zones after development on paper chromatograms. The absorbances of the solutions were determined by means of a Beckman DU spectrophotometer. The results indicated: D-galactose, 61.5% and L-arabinose, 22.8%. A standard determination under the same conditions indicated that L-arabinose and D-galactose were returned in a 96% and 94.1% yield upon acid treatment and quantitization as described above. This adjusts the figures to 65% for D-galactose and 24% for L-arabinose (2:7:1).

This method is far superior to that previously employed using the densitometer method. Although the above return of carbohydrate is quite good it shall be seen that the polysaccharide retained some foreign component even after the base treatment.

5. **The structural elucidation of the arabinogalactan from the holocellulose of green coffee**

a. **The first partially successful series of methylations**

Many attempts were made to fully methylate the above described polysaccharide by a variety of Kuhn's methods (67). Perhaps in this author's hands the procedures were not properly carried out. In this first series of methylations the material was treated first under Haworth conditions (50) using dimethyl sulfoxide and N,N-dimethylformamide.
as the solvents which yielded material with methoxyl content of 30.75% (approx. 2/3). This was followed by three methylations using Kuhn's conditions (67) and the final methoxyl value was not raised above 38% (44.1 theoretical) with loss of practically all the material. The product showed strong carbonyl absorption, very diminished hydroxyl, and aromaticity.

(1) **Isolation of methyl 2,4-di-0-methyl-β-D-galactopyranoside**

The methylated material was hydrolyzed with formic acid and the free sugars subjected to methanolysis. When chromatographed on Silica Gel G layers 5 zones were obtained. When the sirup was chromatographed alongside authentic methyl 2,4-di-0-methyl-β-D-galactopyranoside the slowest moving zone corresponded to it. Eventually crystals were obtained directly from the sirup which were isolated by methanol washing and dried by air and they melted in the range of the above saccharide (73). The remaining zones were isolated by preparative silica gel thin-layer chromatography and hydrolyzed. The hydrolyzed sugars corresponded to known 2,4,6-tri-0-methyl-D-galactose and 2,3,5-tri-0-methyl L-arabinose upon chromatography on microcrystalline cellulose plates.

b. **The second series of methylations**

The arabinogalactan was methylated three times by means of the Haworth reagents (50) with dimethyl sulfoxide and \( \text{N}_{2}\text{N}-\text{dimethylformamide} \) to yield a product of good methoxyl content (36.5%) and in respectable yield. The infrared spectrum showed very diminished hydroxyl and sharp carbonyl absorption. Regardless of how isolated
and methylated, it was apparent that a fully methylated polysaccharide, according to methoxyl analysis, would not be attainable. The structural elucidation was carried out on this product recognizing the fact that some "foreign" substance was present of unknown character.

(1) Hydrolysis and chromatographic investigation using microcrystalline cellulose as a thin-layer adsorbent

The highly methylated polysaccharide was hydrolyzed with formic acid and processed in the usual manner. Paper chromatography revealed three main zones upon development with butanol:ethanol:water, 3:1:1 and indication with aniline phthalate (solvent and indicator used throughout) with the $R_2$ values 1.07, 0.76, and 0.575. Because of these excellent separations a method of isolation utilizing the good differences other than preparative paper chromatograms was desired. Out of this idea the use of microcrystalline cellulose on thin-layer plates was developed. Earlier attempts in this laboratory with other types of cellulose proved to be unworkable. For the research herein described the new method (74,75) proved to be invaluable. The mixture of sugars was loaded on wide (20 x 20 cm.) plates, developed, and a strip in the middle and on both edges indicated to locate the zones, which were scraped off and eluted. The respective three sirups were isolated and subsequently characterized by this method.

(2) The characterization of the three methylated saccharides

(a) 2,6-di-O-methyl-D-galactose

The free sugar was crystallized (from the sirup of $R_2$ 0.575) to a good melting point and the x-ray powder diffraction pattern proven
to be identical with an authentic specimen. The free sugar was further reacted with acidic ethanolic aniline to produce 2,4-di-O-methyl-N-phenyl-D-galactosylamine.

(b) 2,4,6-tri-O-methyl-D-galactose

The sirup ($R_\mu 0.76$) was reacted with acidic ethanolic aniline to form 2,4,6-tri-O-methyl-N-phenyl-D-galactosylamine. This product was recrystallized to an excellent melting point, was proven to be identical with an authentic specimen by x-ray powder diffraction pattern and gave a good methoxyl analysis.

(c) 2,3,5-tri-O-methyl-L-arabinose

The sirup ($R_\mu 1.07$) was reacted with bromine water (66 hr.) and the lactone was isolated by chloroform extraction. The lactone was converted to the crystalline amide by the use of liquid ammonia. The amide exhibited a good melting point, optical rotation, and by x-ray powder diffraction pattern was proven to be identical to authentic 2,3,5-tri-O-methyl-L-arabinonamide.

(3) The determination of relative amounts of methylated sugars in the hydrolyzate

While many authors chose the gas chromatograph, this writer has experienced no difficulty in obtaining the separations but has had trouble in getting integratable peaks. The estimation by this method is still a relatively crude approximation when based on the thermal conductivity of the sugars, hence the densitometer was chosen for this determination. The ratios were established as 5:5:5:8 for 2,3,5-tri-O-methyl-L-arabinose, 2,4-di- and 2,4,6-tri-O-methyl-D-galactose.
From this information the incorrect assumption might be made that every L-arabinofuranose unit accounts for a branch point but the evidence is not conclusive. These two sugars could result from branching as the six position of D-galactose in the chain with D-galactopyranose units finally ending with L-arabinofuranose units as in Figures 1 and 2.

C. Partial Hydrolysis and Subsequent Methylation of the Arabinogalactan

The arabinogalactan was "stripped" of its easily removable arabinose (a common phenomena with the furanose form) by short-term mild acid hydrolysis and methylated. This is a common procedure for opening-up the galactose end groups from attached arabinofuranose groups (if present) first used by White so successfully (29). After three methylations using the highly polar solvents and Haworth's (50) reagents, there was obtained a methylated polysaccharide of 35.2% methoxyl content. Again there was a strong free carbonyl peak in the infrared and the hydroxyl absorption was greatly diminished. Figure 7 displays quite well the picture of what has been taking place during the course of all this methylations and is quite representative of these studies.

(1) Methanolysis and chromatographic investigation

The highly methylated, prehydrolyzed or stripped arabinogalactan (now a galactan) was subjected to a methanolysis and the product was chromatographed on Silica Gel G layers employing 7% methanol in benzene as a developer. The slower moving zones corresponded to those
found earlier from the normal (non-prehydrolyzed) arabinogalactan.
The fastest moving pair of zones were isolated by preparative silica
gel thin-layers.

(2) The isolation of 2,3,4,6-tetra-O-methyl-N-phenyl-D-galactosylamine

The above described sirup (fastest moving pair of zones) was
hydrolyzed with hydrochloric acid, neutralized and reacted with acidic
ethanolic aniline to yield the adduct. The product gave an excellent
melting point, methoxyl analysis, and was found to be identical with
an authentic specimen of 2,3,4,6-tetra-O-methyl-N-phenyl-D-galactosyl-
amine by its x-ray powder diffraction data.

d. General discussion of the arabinogalactan results

In general the structural elucidation of this natural product was
carried out on highly purified (according to effort) but slightly con-
taminated (non-polysaccharide) materials. This could not be avoided,
as there is little doubt in this author's mind that this polysaccharide
carries a residue of difficultly saponifiable phenolic type acids on
its backbone as evidenced by the extreme difficulty encountered by
this author in attaining any degree of full methylation. The other
more convincing aspect is the infrared absorption as illustrated by
Figure 7. The free hydroxyl is almost gone and a strong free carbonyl
is present indicative of an ester function and found in some of the
material before saponification. With all the free hydroxyls present
and many known phenolic acids present in the coffee bean, it seems
very likely that ester combination would occur.
The results of the second series of methylations and the isolation of three crystalline products plus that crystalline product isolated from the prehydrolyzed arabinogalactan establishes the following structure. The isolation of 2,4,6-tri-O-methyl-D-galactose identifies the backbone as that of \((1 \rightarrow 3)\)-\(\beta\)-D-galactopyranose units. The isolation of 2,4-di-O-methyl-D-galactose establishes a branch point at the six position on the D-galactopyranose backbone. The isolation of 2,3,5-tri-O-methyl-L-arabinose establishes its attachment as a furanose side chain attached alone or to a side chain of a few D-galactopyranose units from their end on the six position. This assumption is made from the isolation of 2,3,4,6-tetra-O-methyl-D-galactose after removal of the arabinose.

The structure of this polysaccharide is quite similar to that of the arabinogalactan of Western Larch (Larix occidentalis) as proven by White (29) along with Bouveng and Lindberg (34-39) and illustrated in Figures 1 and 2. They found evidence for some arabinopyranose which was not found in these studies. Almost all investigated arabinogalactans have a common \((1 \rightarrow 3)\)-\(\beta\)-D-galsctopyranose backbone and branching at the six position with arabinofuranose end groups as herein described.
The (10% potassium hydroxide)-insoluble holocellulose of roast coffee earlier isolated by Wolfrom and Laver (13) was dissolved in sulfuric acid (fuming side) diluted, and refluxed until totally hydrolyzed. The quantitative estimation of constituent saccharides in the hydrolyzate was determined by use of a densitometer after the zones were obtained on paper chromatograms. The purpose of this study was to check this holocellulose versus that isolated from green coffee to compare the amounts of the four sugars present. As indicated in Table 2 the results are within experimental error of each other.

The structural elucidation of the green coffee was carried to completion. The mannan was fully methylated and hydrolyzed with formic acid. The main zone was proven to be homogeneous by paper chromatography and electrophoresis. Known 2,3,6-tri-β-methyl-D-mannopyranose 1,4-bis(β-nitrobenzoate) was prepared for comparison purposes. The sugar was proven to be 2,3,6-tri-β-methyl-D-mannose by preparation of the lactone and the above derivative. This establishes the mannan as a linear polymer consisting of (1 → 4)-β-D-mannopyranose units.

The holocellulose of green coffee was selectively hydrolyzed with hot formic acid to yield a product containing 39% D-glucose and a trace of mannose. This residue gave a good yield of α-cellobiose octaacetate. The formic acid insoluble residue was treated with
strong alkali and yielded a high D-glucose content (82%) product which had good cuprammonium hydroxide solubility. The above evidence accounts for cellulose as 5.1% of the total dry weight of the green coffee bean.

The arabinogalactan of green coffee was isolated by several different procedures. Those isolated from readily water-soluble fractions before oxidation and removal of protein were highly complexed with protein. A method was established for removing this protein by extended mixed enzyme system hydrolysis. This polysaccharide fraction was proven to be an arabinogalactan but upon methylation was found to be contaminated with foreign material suspected to be aromatic esters. The holocellulose (oxidized material) was extracted with hot water and after base treatment a polysaccharide was isolated by precipitation from ethanol. This material yielded 65% D-galactose and 24% L-arabinose. Upon repeated methylations the material's methoxyl content could not be raised above 38% (vs. 44% for full methylation). The infrared spectrum suggested the presence of ester functions. The structural elucidation was carried forth on this material and 2,4-di- and 2,4,6-tri-α-methyl-D-galactose were isolated along with 2,3,5-tri-α-methyl-L-arabinose. The polysaccharide was also partially hydrolyzed and methylated. After removing the L-arabinofuranose units, the end group or side chain terminal group was isolated as 2,3,4,6-tetra-α-methyl-D-galactose. This established the polysaccharide as a branched chain structure with the main linear repeating unit to be that of (1→3)-β-D-galactopyranose units with side chains of (1→3)-β-D-galactopyranose units attached in the six position to which terminal L-arabinofuranose units are
attached either directly or through another side chain D-galactose unit. This structure is in general agreement with most of the previously studied arabinogalactans as isolated from wood sources. (Figs. 1 and 2 but without the L-arabinopyranose units).
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I, Donald Leo Patin, was born in West Allis, Wisconsin, on February 29, 1932, and received my primary education at the Holy Assumption parochial school. We moved to a farm in 1946, some 55 miles north of Milwaukee, and I graduated from Campbellsport High School in 1950. From 1951 to 1955 I was in service with the United States Navy, which included a tour of combat in Korean waters. I was honorably discharged as a Petty Officer Second Class specializing in Navigation and Visual Signaling. I took the hand of Donna M. Hatch in marriage in 1953. In March 1955 I commenced my higher education at Wisconsin State College at River Falls where I received the Bachelor of Science degree with a major in chemistry and a minor in mathematics in June 1958. I enrolled in the Graduate School of The Ohio State University in September 1958 and was employed as a Teaching Assistant by the Department of Chemistry. From June 1959 to September 1960 I held a Research Fellowship sponsored by Westreco Inc. (O.S.U.R.F. 878). In October 1960 I was appointed Research Associate, a full-time position with Professor Wolfson to assist with administrative matters in addition to the research herein described, a position which I hold at this time. Our marriage has been blessed with four children; Douglas 10, Kathleen 9, John 5, and Mary 4.