APIOGALACTURONANS FROM THE CELL WALL OF LEMNA MINOR L.

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DAVID A. HART
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This is to certify that the

thesis entitled

APIOGALACTURONANS FROM THE CELL WALL
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David A. Hart

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Paul K. Kindel
Major professor

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ABSTRACT

APIOGALACTURONANS FROM THE CELL WALL OF LEMNA MINOR L.

By David A. Hart

A mild, reproducible procedure has been developed for the isolation of D-apiose-containing polysaccharides from the cell wall of Lemna minor. The procedure is based on the extraction of the cell walls with 0.5% ammonium oxalate. The conditions used at 22° have no known degradative effect on polysaccharides. On a dry weight basis, the polysaccharides extracted with ammonium oxalate made up 14% of the material designated cell walls and contained 20% of the D-apiose originally present in the cell walls. The cell walls, as isolated, contained 83% of the D-apiose present in L. minor. After extraction with ammonium oxalate, purified polysaccharides were obtained by DEAE-Sephadex column chromatography and by fractional precipitation with sodium chloride. With these procedures, the material extracted at 22° could be separated into at least five polysaccharides. On a dry weight basis, two of these polysaccharides made up more than 50% of the material extracted at 22°. There was a direct relationship between the D-apiose content of the polysaccharides and their solubility in sodium chloride solutions; those of highest D-apiose content were most soluble. All of the polysaccharides

isolated appeared to be of one general type, namely galacturonans to which were attached sidechains containing D-apiose. The D-apiose content of the apiogalacturonans varied from 7.9 to 38.1%. The content of esterified D-galacturonic acid residues in all apiogalacturonans was low, being in the range 1.0-3.5%. Hydrolysis of a representative apiogalacturonan with dilute acid resulted in the complete removal of the D-apiose with little or no degradation of the galacturonan portion. Treatment of polysaccharide fractions with pectinase established that those of high D-apiose content and soluble in 1.0 M-sodium chloride were not degraded whereas those of low D-apiose content and insoluble in 1.0 M-sodium chloride were extensively degraded. When the D-apiose was removed from a typical pectinase-resistent polysaccharide, the remainder of the polysaccharide was readily degraded by this enzyme. Periodate oxidation of representative polysaccharide fractions and apiogalacturonans and determination of formaldehyde released, showed that about 50% of the D-apiose molecules were substituted at either the 3- or the 3'-position.

The apiogalacturonans, as the sodium salts, were partially degraded when heated under mildly acidic conditions. The extent of the hydrolysis under these conditions was approximately equal to the percent D-apiose of the apiogalacturonans. The same two degradation products were obtained from all of the apiogalacturonans. These were D-apiose and a disaccharide of D-apiose named apibiose. The residues from

the degradation, the galacturonans, were not characterized. Periodate oxidation of apibiose and crystalline apibiose phenylosotriazole and determination of formaldehyde released, showed that the position of the linkage between the two D-apiose molecules was 1→3'. Proton magnetic resonance spectrometry and molecular rotational data suggested that the linkage had the β configuration. Methylation analysis of apibiose and apibiose phenylosotriazole indicated that the non-reducing terminal D-apiose molecule had the D-apio-D-furanose configuration. The configuration at C-3 of the reducing terminal D-apiose molecule was not determined. Therefore the disaccharide is O-β-D-apio-D-furanosyl-(1→3')-D-apiose. When attached as sidechains to the α-(1→4)-galacturonan, the disaccharide is (2 and/or 3)-O-[O-β-D-apio-D-furanosyl-(1→3')-(α or β)-D-apio-(D or L)-furanosyl]-galacturonan.

APIOGALACTURONANS FROM THE CELL WALL OF *LEMNA MINOR* L.

By

David A. Hart

A THESIS

Submitted to
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DOCTOR OF PHILOSOPHY

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Dedicated to

My Wife and Parents

VITA

The author was born, raised and educated in Marquette, Michigan. He was born in 1942, graduated from J. D. Pierce High School in 1960 and graduated from Northern Michigan University with a B.A. degree in 1964. In the Fall of 1964 he then entered graduate school at Michigan State University. After stealing food from the ducks for five years, he is presently severing ties with Lower Michigan and departing for the Windy City where he will join the laboratory of Dr. A. Nisonoff.

ACKNOWLEDGMENTS

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The author is particularly grateful to his wife, Mary, for her continued encouragement and for her work as an educator of the masses to help finance this undertaking. The financial support of a National Aeronautics and Space Administration Fellowship is also gratefully acknowledged.

ORGANIZATION OF THE THESIS

The thesis is divided into three parts. Part 1 is a brief literature review of the chemistry of apiose. Parts 2 and 3 are related but deal with separate aspects of the problem. Each of these parts has a separate Introduction, Materials and Methods, Results and Discussion. However all of the references have been placed at the end of Part 3.

Part 2 has been accepted for publication in Biochemical Journal. Part 3 has been written in the same form as Part 2 but will be rewritten for submission to a different journal.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SCHEMES

PART 1

THE CHEMISTRY OF APIOSE

The Chemistry of Apiose

In 1901, Vongerichten reported that an unknown sugar was released from the flavonoid glycoside apiin by mild acid hydrolysis. The sugar did not yield furfural on heating with concentrated acid. Vongerichten prepared two crystalline derivatives of the sugar, the phenylosazone and the p-bromophenylosazone. He concluded that the sugar was a pentose and named it apiose, after the Linnean name of the parsley plant, Apium petroselium L.

A year later, Vongerichten (1902) reported the further characterization of the apiose by preparation of apionic acid from apiose by oxidation with bromine water. This compound was converted to a crystalline strontium salt, an amorphous calcium salt and a crystalline phenylhydrazide. Reduction of calcium apionate with hydriodic acid and phosphorus resulted in a volatile acid which was identified as isovaleric acid. He therefore concluded that apiose was a branched-chain pentose (I).

In 1906, Vongerichten and Müller prepared another crystalline derivative of apiose, the α-benzyl-α-phenylhydrazone. Regeneration of the free sugar from this crystalline derivative led to the first isolation of a pure syrup of apiose.

Information about the stereochemistry of apiose was first obtained by Schmidt (1930). He confirmed Vongerichten's work and elucidated the configuration at C-2 by application

of the rule for determining the configuration of the α-carbon of α-hydroxycarboxylic acids to apionic acid. He concluded that the apionic acid was D-apionic acid (II) and therefore the apiose from apiin was D-apiose (3-C-hydroxymethyl-aldehydo-D-glycero-tetrose, III).

D-Apiose is unusual among pentoses because when it cyclizes it can only form a furanose ring. Furthermore, cyclization results in the formation of two new asymmetric centers at C-1 and C-3. This leads to the possibility of four cyclic isomers for D-apiose, two α-isomers (IV and V) and two β-isomers (VI and VII). In solution these are in equilibrium with a fifth isomer, the aldehydo form. The nomenclature for the cyclic isomers has not been officially established. However, it has been suggested by Cahn (in Bell, Isherwood and Hardwick, 1954) that the following nomenclature be employed. The designation "D-apio" should refer to only the configuration at C-2. When a furanose ring is formed, the stereochemistry at C-3 should be designated with a second D- or L-, depending on whether the hydroxymethyl group at C-3 is trans or cis, respectively, to the hydroxyl group at C-2. Using such a system, the structure (IV) is designated α-D-apio-D-furanose. Only members of the D-series have been described since this is apparently the naturally occuring isomer. However, there are also four possible cyclic isomers for the L-series, for a total of eight cyclic isomers for apiose.

Both D-apiose (Gorin and Perlin, 1958, Khalique, 1962, and Williams and Jones, 1964) and L-apiose (Schaffer, 1959, and Weygand and Schemiechen, 1959) have been synthesized chemically.

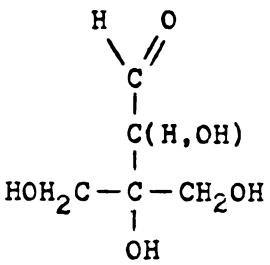
There have been several numbering systems employed by different authors for the carbons of apiose. In this thesis, the carbons will be numbered as shown in (VIII).

Recently, the fine structure of certain naturally-occuring D-apiose containing compounds and certain derivatives of D-apiose has been elucidated. The results of several workers have led to the elucidation of the structure of apiin. After methylation of apiin and hydrolysis, Hemming and Ollis (1953) isolated a tri-O-methyl derivative of D-apiose and 3,4,6-tri-O-methyl-D-glucose. From this data, they concluded that the linkage between the two sugars was from C-1 of the D-apiose to C-2 of the D-glucopyranosyl moiety. Halyalker, Jones and Perry (1965) investigated the stereochemistry at C-1 and C-3 of the D-apiose moiety. They also methylated apiin and isolated the resulting tri-O-methyl-D-apiose. The compound was identical by paper, thin layer and gas-liquid chromatography, optical rotation, and infrared spectra, to chemically synthesized 2,3,3'-tri-O-methyl-D-apio-D-furanose. Periodate oxidation data also indicated that the hydroxyl groups at C-2 and C-3 were in the <u>cis</u> configuration. Employing the molecular rotation theory of Klyne (1950), these workers concluded

that the stereochemistry of the linkage between C-1 of the D-apiose and C-2 of the D-glucose was β. Therefore apiin is 7-O-[O-β-D-apio-D-furanosyl-(1–2)-β-D-glucopyranosyl]-apigenin (IX).

Hattori and Imaseki (1959) have characterized a D-apiose containing phenolic glycoside (furcatin) from Viburum furcatum Blume. From partial acid and enzymatic hydrolysis of this compound, plus periodate oxidation, they concluded that the structure of the carbohydrate portion of the molecule was O-[O-(α or β)-D-apio-(D or L)-furanosyl-(1–6)-β-D-glucopyranosyl]-p-vinylphenol (X). They did not determine the stereochemistry at C-3 of the D-apiose nor did they present evidence for the glycosidic linkage between D-apiose and D-glucose. Imaseki and Yamamoto (1961) have isolated an enzyme system from the same plant which specifically hydrolyzes the disaccharide: aglycon linkage.

The isopropylidene derivatives of apiose have been studied in detail. Williams and Jones (1964) have synthesized the 1,2:3,3'-di-O-isopropylidene derivatives of both D- and L-apiose. The L-isomer had an infrared spectrum which was different from that obtained for the D-isomer. The authors concluded that the difference may be due to a difference in the configuration at C-3 in the two compounds, although the structures of the two were not determined. Using D-apiose obtained from acid hydrolysates of Zostera marina, Carey, Ball, and Long (1966) have shown that the



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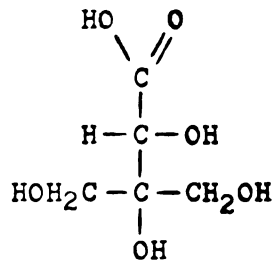

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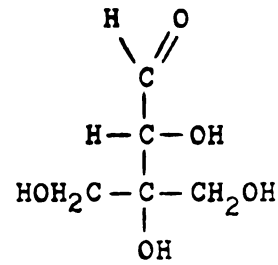

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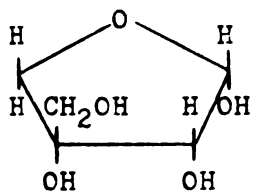

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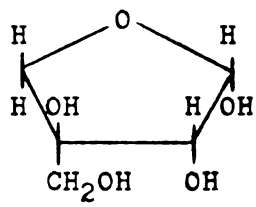

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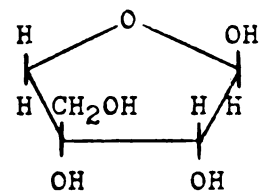

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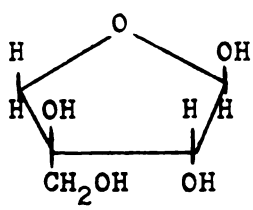

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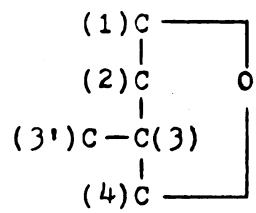

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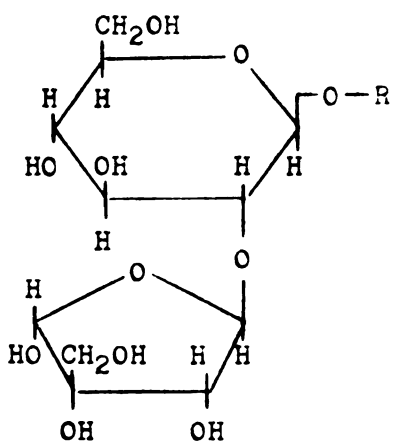

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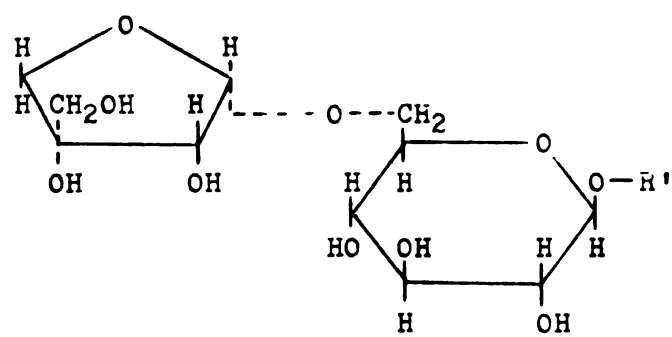


(X)

List of Structures. The dotted lines indicate undetermined stereochemistry. The non-carbohydrate components of compounds are indicated by R and R'.

predominant di-O-isopropylidene derivative obtained is 1,2:3,3'-di-O-isopropylidene-α-D-apio-L-furanose. Graded acid hydrolysis led to the isolation of the 1,2-isopropylidene derivative of the same furanose form. This result in no way indicates that D-apiose exists in this form in Zostera marina. Ball, Carey, Klundt, and Long (1969) have also isolated and characterized both the 1,2:3,3'-di-O-isopropylidene and the 1,2-O-isopropylidene of D-apio-D-furanose.

Long's group have prepared derivatives of D-apiose in which the ring oxygen has been replaced with a nitrogen atom (Halford, Ball, and Long, 1969) or a sulfur atom (Halford, Ball, and Long, 1968). This group has prepared several crystalline derivatives and intermediates of the compounds described above which they have also characterized.

Additional literature dealing with the chemistry and biochemistry of D-apiose is cited in the remainder of the thesis where specific problems are discussed.

PART 2

ISOLATION AND PARTIAL CHARACTERIZATION OF APIOGALACTURONANS

INTRODUCTION

The branched-chain aldopentose D-apiose (3-C-hydroxymethyl-aldehydo-D-glycero-tetrose) has been identified as a constituent of a large variety of plants. In some plants it exists as a component of a flavone, isoflavone or phenolic glycoside such as apiin (Vongerichten, 1901; Nakaoki, Morita, Motosune, Hiraki and Takeuchi, 1955; Wagner and Kirmayer, 1957; Rahman, 1958), lanceolarin (Malhotra, Murti and Seshadri, 1956) or furcatin (Hattori and Imaseki, 1959). However, in Posidonia australis (Bell, Isherwood and Hardwick, 1954), Tilia sp. (Bacon, 1963), Zostera marina (Bacon, 1963; Williams and Jones, 1964; Ovodova, Vaskovsky and Ovodov, 1968), Lemna gibba (Beck and Kandler, 1965), L. minor (Duff, 1965; Beck and Kandler, 1965; Mendicino and Picken, 1965; Beck, 1966, 1967), Z. nana (Duff, 1965), Z. pacifica and Phyllospadix (Ovodova et al., 1968) it appears to be primarily a component of polysaccharides. In the case of Z. marina (Williams and Jones, 1964; Ovodova et al., 1968), Tilia sp. (Bacon, 1963), L. minor (Duff, 1965; Mendicino and Picken, 1965; Beck, 1966, 1967), Z. pacifica and Phyllospadix (Ovodova et al., 1968) a polysaccharide fraction or fractions were isolated and shown to contain D-apiose. Beck (1967) and Ovodova et al., (1968) partially characterized their polysaccharides.

In a study of L. minor (duckweed), one of the richest known sources of D-apiose, Duff (1965) showed that more than

90% of the D-apiose present was not extracted by organic solvents. Extraction of the insoluble material with a succession of alkaline conditions indicated that the bound D-apiose was continuously extracted and that no single fraction contained a majority of the D-apiose. From a study of the incorporation of $^{14}CO_2$ into the D-apiose of L. minor and L. gibba under various conditions, Beck and Kandler (1965) concluded that the D-apiose was not part of a storage material but rather a component of the cell wall. While the present study was in progress, Beck (1966, 1967) reported the isolation of two apiogalacturonans from L. minor which contained 28% and 25% D-apiose, one of which contained, in addition, D-xylose and D-galactose.

We are interested in the isolation of plant polysaccharides containing D-apiose in order to determine the structure of such unique polysaccharides and to study the mechanism and control of their biosynthesis. Attainment of these goals depended on the development of reproducible methods for their isolation and fractionation. In addition, the methods should result in minimal degradation of the polysaccharides. This precluded the use of alkaline extractions because of the degradative effect of such conditions on polysaccharide material (Whistler and BeMiller, 1958; Neukom and Deuel, 1958). It also precluded the use of acidic conditions due to the acid lability of the apiofuranoside glycosidic linkage (Vongerichten, 1901; Mendicino

and Picken, 1965). Furthermore, even near neutrality, high temperatures must be used with caution since pectins may be degraded by a β-elimination reaction (Albersheim, 1959; Albersheim, Neukom and Deuel, 1960).

We therefore examined procedures which have been used to extract pectic substances since it is known that their extraction may be facilitated by relatively mild conditions. When such conditions were applied to L. minor cell walls, polysaccharides rich in D-apiose were solubilized. The present study describes the isolation and partial characterization of these polysaccharides.

MATERIALS AND METHODS

Materials

L. minor was obtained from the Battle Creek River, Bellvue, Michigan. The plant material was washed extensively and either used immediately or stored at -20°. D-Apiose was obtained from once recrystallized apiin. Crystalline apiin was isolated from Petroselinum crispum (parsley) seeds by the method of Gupta and Seshadri (1952) and hydrolyzed with 0.1 N-sulfuric acid for 30 min. at 100° (Vongerichten, 1901). Pure D-apiose was isolated by partition column chromatography on acid-washed Celite 535 using the procedure of Lemieux (1962).

[U-^{14}C] D-Apiose was prepared from UDP-[U-^{14}C] D-glucuronic acid (New England Nuclear Corporation, Boston,

Mass., U.S.A.). The procedure of Gustine and Kindel (1969) was used to convert UDP-[U-^{14}C] D-glucuronic acid to Compound III. Compound III was the designation given by these workers to the D-apiose-containing compound obtained by their procedure. After paper chromatography in solvent E, Compound III was chromatographed in solvent D. For the preparation of free, radioactive D-apiose, Compound III was hydrolyzed in 0.2 N-sulfuric acid for 90 min. at 100° and the hydrolysate treated as described previously (Gustine and Kindel, 1969). Before hydrolysis, sufficient non-radioactive D-apiose was added to give the specific radioactivity indicated below. The free [U-^{14}C] D-apiose from the hydrolysis was chromatographed on paper in solvents A, B and C and in each it migrated as a single radioactive peak. The specific radioactivity was determined by measuring sugar using Nelson's method (1944) with an appropriate elution blank and radioactivity using liquid scintillation counting in Bray's solution (1960). The specific radioactivity of the [U-^{14}C] D-apiose was 68160 disintegrations/min./μmole of D-apiose. The radioactive D-apiose was designated [U-^{14}C] D-apiose since it was derived from the glucuronic acid portion of UDP-[U-^{14}C] D-glucuronic acid.

Purified Fungal pectinase was purchased from Sigma Chemical Company, St. Louis, Mo., U.S.A. The enzyme was purified 30-fold over the crude preparation by the manufacturer who stated it still contained several other enzymes.

The specific activity of this batch (Lot 125B-0350) as measured by Sigma was 0.7 units per mg. of solid. A unit was defined as that amount of enzyme which liberated 1 umole of D-galacturonic acid per min. at 25° and at pH 4.0 with de-esterified citrus pectin as the substrate.

General Methods

Solutions were concentrated under reduced pressure by rotatory evaporation at temperatures less than 35°. Polysaccharide material was desiccated to constant weight in vacuo and over phosphorus pentoxide except in Table 2. Calcium chloride and silver nitrate were used to test for oxalate and chloride ions, respectively. Centrifugations were done at 4°. Radioactivity on chromatograms was detected with a Packard radiochromatogram scanner, model 7201 (Packard Instrument Co., Downers Grove, Ill., U.S.A.). All other measurements were made in the scintillation solution of Bray (1960) with a Packard Tri-Carb liquid scintillation counter, Model 3310. Optical rotations were determined with a Zeiss Photoelectric Precision Polarimeter 0.005° (Carl Zeiss, Oberkochen, Wuerttemberg, Germany) at 22° with a polarimeter tube having a 1 cm. light path and light of 578 mμ (Hg) wavelength. Polysaccharides were dissolved in 0.067 M-potassium dihydrogen phosphate-disodium hydrogen phosphate, pH 7.7, unless otherwise noted, and their concentrations ranged from 9.1-21 mg./ml.

Paper Chromatography

Descending paper chromatography was used and was carried out with Whatman No. 3MM paper prewashed with 0.1 M-citric acid followed by distilled water. The following solvents were employed: A) ethyl acetate-water-acetic acid-formic acid (18:4:3:1, by vol.), B) propan-2-ol-water (9:1, by vol.), C) butan-1-ol-acetic acid-water (4:1:5, by vol., upper phase), D) propan-1-ol-ethyl acetate-water (7:1:2, by vol.), E) 95% aqueous ethanol-1.0 M-ammonium acetate, pH 7.5 (7:3, by vol.), F) pyridine-ethyl acetate-acetic acid-water (5:5:1:3, by vol.). Sugars were detected on chromatograms by spraying with aniline hydrogen phthalate (Partridge, 1949) or by using the silver nitrate dip method (Trevelyan, Proctor and Harrison, 1950).

DEAE-Sephadex Column Chromatography

Column chromatography of the five polysaccharide fractions (see the fractionation experiment in the Results) was carried out on DEAE-Sephadex (A-25 medium, capacity: 3.5 m-equiv./g., 100-270 mesh, Pharmacia Fine Chemicals, Inc., Uppsala, Sweden). Before use, the DEAE-Sephadex was repeatedly suspended in 0.067 M-potassium dihydrogen phosphate-disodium hydrogen phosphate buffer, pH 7.7, and the fines were removed. Before a polysaccharide fraction was applied, the column was washed with 2-3 bed volumes of the phosphate buffer. The polysaccharide fractions were dissolved in either water or the same buffer and applied to

the column at approximately the same rate as the column was to be operated. After a washing with the phosphate buffer, the polysaccharides were eluted with a step gradient of 0.1-0.3 M-sodium chloride in the phosphate buffer. The individual polysaccharides were obtained by pooling the appropriate fractions from the columns and dialyzing them against distilled water until negative for chloride ion. The non-diffusible material was then lyophilized and desiccated.

Before characterization, some polysaccharides were converted to the hydrogen form by passage through a column of Dowex 50W-X8 (50-100 mesh, H^+ form). The polysaccharide solutions were then lyophilized and desiccated.

Analysis of DEAE-Sephadex Column Fractions for Uronic Acid and D-Apiose

The column fractions were assayed for uronic acid by a modification of the method of Dische (1962). One-half (0.5) ml. samples or appropriate samples diluted to 0.5 ml. with water in test tubes were cooled to 4°. On top of each sample was layered 0.2 ml. of 0.1% (w/v) carbazole (recrystallized once from benzene) in ethanol. The tubes were cooled again to 4° and then 6.0 ml. of 15.8 M-sulfuric acid was added. The solutions were thoroughly mixed with a Vortex Jr. mixer (Scientific Industries, Queens Village, N.Y., U.S.A.) at 22°, heated at 100° for 20 min., cooled in water to 22° and the extinction at 525 mμ determined immediately. With these conditions L-arabinose, D-xylose, and

D-apiose gave 3.4%, 4.8%, and 2.4% respectively of the color obtained with D-galacturonic acid. In the above procedure, the samples were heated after the addition of carbazole. This is in contrast to the original Dische procedure and has two advantages; first, the sensitivity of the method is increased, and second, the extinction readings can be made immediately after the 20 min. heating period (Gauthier and Kenyon, 1966).

Early in this work it was discovered that the 22° and 70° sodium chloride-soluble and -insoluble polysaccharide fractions quantitatively released their D-apiose on mild acid hydrolysis without the release of significant amounts of other reducing material. This property was used to develop the following assay for D-apiose in the column fractions. A 1.0 ml. sample or an appropriate aliquot diluted to 1.0 ml. with water was mixed with 0.1 ml. of 1.0 N-hydrochloric acid and heated at 100° for 30 min. The samples were cooled and the liberated reducing material was determined by the method of Nelson (1944). The extinctions were read at 540 mμ.

Quantitative Determination of Sugars in Polysaccharide Material

The percent D-galacturonic acid was determined, after saponification (McComb and McCready, 1952), by the above described modification of the sulfuric acid-carbazole method.

D-Apiose was determined by isotope dilution. For

each determination, approximately 5-10 mg. of polysaccharide material was suspended in 1.0 ml. of [U-^{14}C] D-apiose containing 9474 disintegrations/min. and 1.0 ml. of 1 N-sulfuric acid was added. The solution was heated at 100° for 1 hr., cooled to 22°, neutralized to pH 6-7 with sodium hydroxide, and then nine volumes of absolute ethanol was added. The resulting precipitate was removed by centrifugation. The supernatant solution was decanted, concentrated, streaked on paper and the chromatogram was developed in solvent B. A strip of the chromatogram was treated by the $AgNO_3$ dip method. The material which cochromatographed with the radioactive D-apiose was eluted and restreaked on paper and the chromatogram developed in solvent C. After elution, the specific radioactivity of the radioactive D-apiose was determined by measuring reducing sugar and radioactivity as described above. The amount of D-apiose released from the polysaccharide material was calculated from the percentage of the total radioactive [U-^{14}C] D-apiose recovered and the specific radioactivity of the isolated and added [U-^{14}C] D-apiose.

Determination of the Content of Esterified D-Galacturonic Acid Residues in Polysaccharide Fractions

The methoxyl content of polysaccharide fractions was determined by the method of Schultz (1965). Polysaccharide fractions (20-50 mg.) were dissolved in carbon dioxide-free water, titrated to a phenolphthalein endpoint with 0.005

N-carbonate-free sodium hydroxide, and then 5-10 ml. of 0.1 N-carbonate-free sodium hydroxide was added. The solutions were kept at 22° for 30 min. and then an equal volume of 0.1 N-hydrochloric acid was added. The solutions were titrated to a phenolphthalein endpoint under nitrogen with the 0.005 N-sodium hydroxide. With this method, a value of 8.3% was obtained for the methoxyl content of citrus pectin (grade II, Sigma Chemical Company).

The percent of esterified D-galacturonic acid residues in the polysaccharide fractions was calculated from the percent D-galacturonic acid, obtained from the sulfuric acid-carbazole test, and the methoxyl content obtained as described above.

Preparation of Partially Hydrolyzed Polysaccharide

D-Apiose was removed from the 22° sodium chloride-soluble fraction by hydrolysis with 0.1 N-hydrochloric acid for 30 min. at 100°. The solution was cooled, neutralized with sodium hydroxide to pH 7, and then dialyzed against distilled water until the diffusate was negative for chloride ion. The non-diffusible material was lyophilized and stored over phosphorus pentoxide *in vacuo*. This material was used in the pectinase experiment depicted in Figure 5.

Pectinase Hydrolysis of Polysaccharide Fractions

Polysaccharide fractions were incubated with pectinase at 37° in 0.025 M-sodium acetate buffer, pH 4.5. The enzyme

was dissolved in 0.05 M-sodium acetate buffer, pH 4.5, and the polysaccharide fractions were dissolved in distilled water. The reaction was followed by measuring the increase in reducing material, as determined by the method of Nelson (1944), with time. The extinctions were read at 540 mμ.

Periodate Oxidation of Polysaccharide Materials and Release of Formaldehyde

Polysaccharide material (approx. 5.0 mg.) was dissolved in 1.0 ml. of 0.05 M-sodium acetate buffer, pH 5.0, and then 1.0 ml. of 0.1 M-sodium metaperiodate was added. The solution was kept at 22° for 2 hr. in the dark. Preliminary experiments showed that the release of formaldehyde was virtually complete after 1 hr. Formaldehyde was determined using chromotropic acid (Speck, 1962). The chromotropic acid was recrystallized once from aqueous 50% (v/v) ethanol.

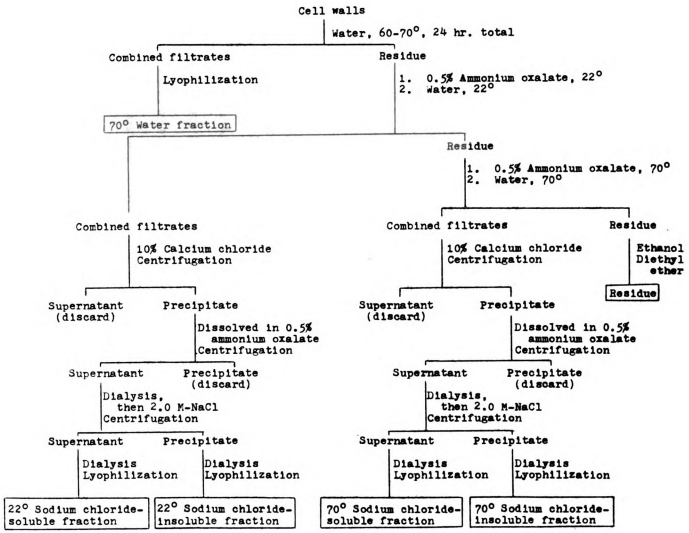
RESULTS

Extraction and Fractionation of L. minor

The L. minor plants were initially extracted so that cell walls were isolated. The cell walls were fractionated by the procedure outlined in Scheme 1. A typical fractionation experiment is described below.

Fresh L. minor (350 g., 24.15 g. dry weight) was homogenized for 1 min. periods in a Waring Blendor successively with 1.0 M-sodium chloride (3 times), water (1 time),

Scheme 1. Flow sheet outlining the procedure for fractionating L. minor cell walls.


Cell walls
Water, 60-70°, 24 hr. total
Combined filtrates
Residue
Lyophillization
1. 0.5% Ammonium oxalate, 22°
2. Water, 22°
70° Water fraction
Residue
1. 0.5% Ammonium oxalate, 70°
2. Water, 70°
Combined filtrates
Combined filtrates
Residue
10% Calcium chloride
Centrifugation
10% Calcium chloride
Centrifugation
Ethanol
Diethyl
ether
Residue
Supernatant
(discard)
Precipitate
Dissolved in 0.5%
ammonium oxalate
Centrifugation
Supernatant
(discard)
Precipitate
Supernatant
Precipitate
(discard)
Dialysis,
then 2.0 M-NaCl
Centrifugation
Supernatant
Precipitate
Supernatant
Precipitate
Dialysis
Lyophilization
Dialysis
Lyophilization
Dialysis
Lyophilization
Dialysis
Lyophilization
22° Sodium chloride-
soluble fraction
22° Sodium chloride-
insoluble fraction
70° Sodium chloride-
soluble fraction
70° Sodium chloride-
insoluble fraction

0.1 M-sodium chloride (2 times), and water (3 times), using 1 l. quantities for each homogenization. The suspension was filtered through six layers of cheesecloth after each homogenization. The final residue was washed with water until the washings were free of chloride ion, dried by solvent exchange with aqueous 95% (v/v) ethanol, absolute ethanol, absolute ethanol-diethyl ether and diethyl ether and desiccated to yield 7.97 g. of white material, designated cell walls.

Cell walls, 7.50 g., were suspended in 1 l. of water and stirred at 60-70° for 8 hr. The suspension was filtered by suction and the process was repeated twice. The combined filtrates were concentrated, lyophilized, and desiccated to yield 280.6 mg. of brown material. This was called the 70° water fraction.

The residue was suspended in 1 l. of 0.5% (w/v) ammonium oxalate, pH 6.2, and stirred at 22° for 3 hr. The suspension was filtered and the process was repeated. The residue was then extracted with 1 l. of water at 22° for 3 hr. and the suspension filtered. The extraction of the residue with water was repeated once and the four filtrates were combined and concentrated to approximately 400 ml. The solution was dialyzed against distilled water until the diffusate was negative for oxalate ion. The solution of non-diffusible material was transferred to a beaker and 10% (w/v) calcium chloride was added slowly to the stirred

solution until no further precipitation occurred. After cooling to 4°, the suspension was centrifuged at 35000 x g. for 15 min. and the supernatant solution was decanted and discarded. With stirring, the precipitate was redissolved in approximately 400 ml. of 0.5% (w/v) ammonium oxalate at 22°. After cooling to 4°, the insoluble calcium oxalate was removed by centrifugation at 35000 x g. for 20 min. and discarded. After decanting, the supernatant solution was dialyzed against distilled water until the diffusate was negative for oxalate ion. The solution of non-diffusible material was concentrated to 300 ml. and an equal volume of 2.0 M-sodium chloride was added dropwise to the constantly stirred solution. After cooling to 4°, the suspension was centrifuged at 35000 x g. for 15 min. The supernatant solution was concentrated to about 250 ml. and then dialyzed against distilled water until the diffusate was negative for chloride ion. No further precipitation of polysaccharides occurred during this reduction in volume even though the concentration of the sodium chloride in the supernatant solution increased to at least 2 M. The non-diffusible material was lyophilized and desiccated to yield 371 mg. of white material. This material was designated the 22° sodium chloride-soluble fraction. The material precipitated by the 1.0 M-sodium chloride was resuspended in water and dialyzed against distilled water until the diffusate was negative for chloride ion. The solution of non-diffusible material was lyophilized and desiccated to yield 321 mg. of

white material. This material was designated the 22° sodium chloride-insoluble fraction.

The residue from the 22° extraction procedure was further extracted with 1 l. of 0.5% (w/v) ammonium oxalate, pH 6.2, at 70° for 3 hr. The suspension was filtered and the process was repeated. The residue was then extracted with 1 l. of water at 70° for 3 hr. and the suspension filtered. The extraction of the residue with water was repeated once and the four filtrates were combined. The material solubilized by the 70° extraction procedure was taken through the same steps as that solubilized by the 22° extraction procedure. The material designated the 70° sodium chloride-soluble fraction weighed 211 mg. and was yellow, while that designated the 70° sodium chloride-insoluble fraction weighed 105 mg. and was white.

The residue from the 70° extraction was dried by solvent exchange as described above for the cell walls. It was then desiccated to yield 5.15 g. of material. The results of this fractionation experiment are summarized in Table 1.

In a separate experiment, the efficiency of the individual ammonium oxalate and subsequent water extractions was examined. The results are shown in Table 2.

Table 1. Fractionation of L. minor

The weights of the six fractions obtained from the cell walls have been calculated on the basis that 7.97 g. of cell wall material was fractionated.

Fraction	Amount obtained		D-Apiose content	D-Apiose recovered in each fraction	
	(g.)	(%)	(%)	(%)*	(%)+
Dry plants	24.15	---	5.8	100	---
Cell wall	7.97	100	14.6	83.1	100
70° Water	0.298	3.7	3.1	0.7	0.8
22° Ammonium oxalate extraction					
Sodium chloride-soluble	0.395	5.0	33.1	9.3	11.2
Sodium chloride-insoluble	0.341	4.3	10.1	2.5	3.0
70° Ammonium oxalate extraction					
Sodium chloride-soluble	0.224	2.8	21.6	3.5	4.2
Sodium chloride-insoluble	0.112	1.4	14.0	1.1	1.3
Residue	5.47	68.6	16.2	63.3	76.2
		85.8			96.7

*Values calculated on the basis of the D-apiose content of the dried plants.

+Values calculated on the basis of the D-apiose content of the dried cell walls.

Table 2. Efficiency of the ammonium oxalate extractions

L. minor cell walls were extracted with water at 70° as described in the text and dried. This material (3.8 g.) was further extracted as described in the text. For each extraction, 400 ml. of extractant was used. The individual extracts were concentrated at 30°, dialyzed against distilled water, lyophilized and weighed.

Extractant	Amount extracted	
	mg.	%
0.5% Ammonium oxalate, 22°	335.1	8.8
0.5% Ammonium oxalate, 22°	24.4	0.6
Water, 22°	18.8	0.5
Water, 22°	11.3	0.3
0.5% Ammonium oxalate, 70°	89.2	2.4
0.5% Ammonium oxalate, 70°	26.2	0.7
Water, 70°	27.5	0.7
Water, 70°	2.1	0.1
Residue	3200	84.2

Column Chromatography of Polysaccharide Fractions on DEAE-Sephadex

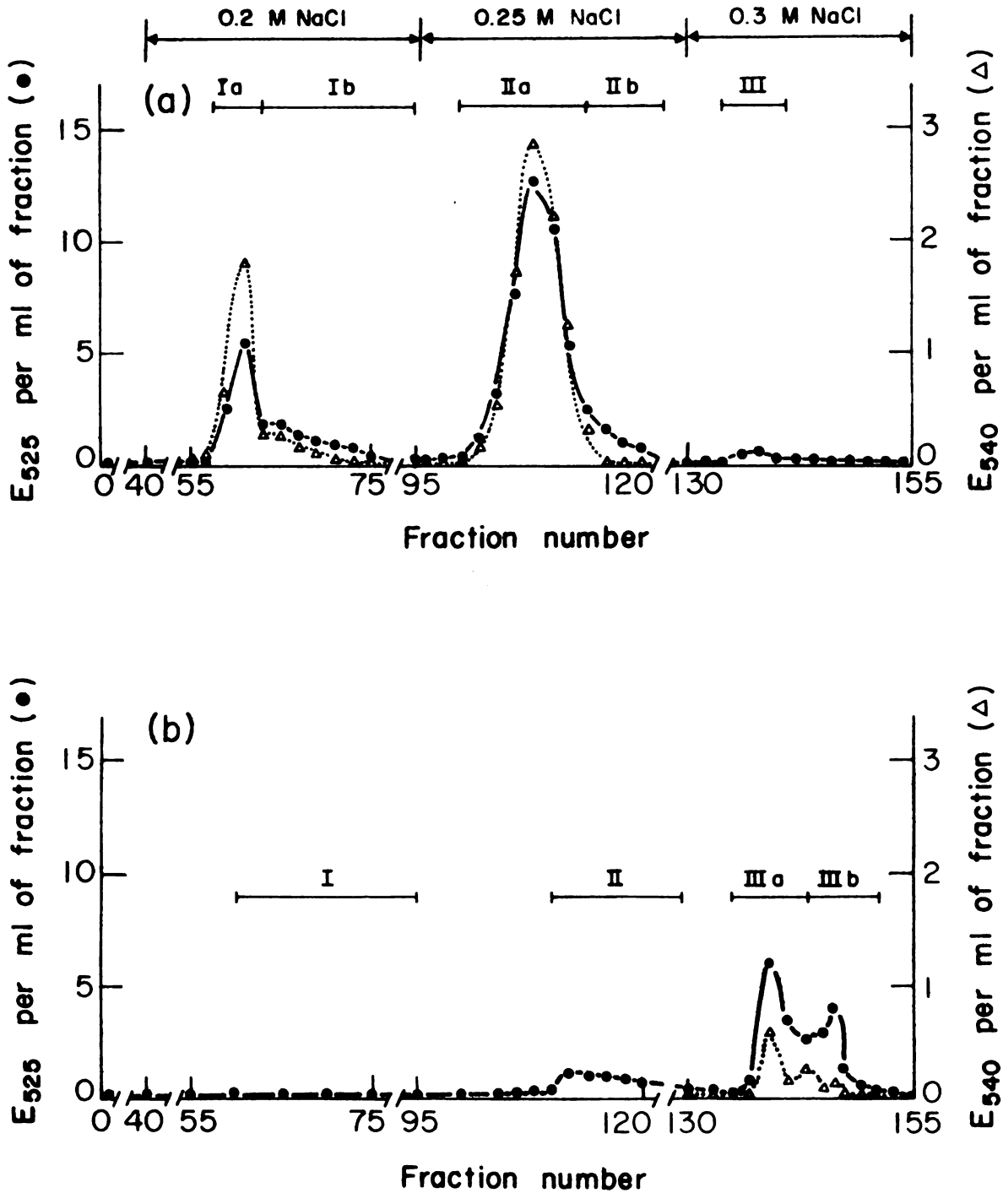
The five fractions obtained from the fractionation experiment described in the preceding section were subjected to column chromatography on DEAE-Sephadex. The procedure described in the Materials and Methods section was followed.

The 70° water fraction (110 mg.) was suspended in 35 ml. of water and stirred several hours, first at room temperature and then at 60-70°. Since all the material did not dissolve, the suspension was centrifuged at 35000 x g. for 15 min. The supernatant solution was decanted and the precipitate was dried with acetone and weighed (13.2 mg.). The material in the supernatant solution (96.8 mg.) was applied to a DEAE-Sephadex column (2.2 cm. i.d. x 25 cm.). Fractions were collected at a rate of 0.20 ml./min. No material was eluted which reacted in the tests for uronic acid and D-apiose. The top of the column contained a yellow band which did not elute. Because of its low D-apiose content, further attempts to characterize this fraction were not made.

The 22° sodium chloride-soluble fraction (335.2 mg.) was dissolved in 20 ml. of the phosphate buffer and chromatographed on a DEAE-Sephadex column. The elution profile is given in Figure 1a. The indicated fractions were pooled and the polysaccharides were isolated as described in the

Figures 1a and 1b. Column chromatograms of 22° sodium chloride-soluble and -insoluble fractions.

Figures 1a and 1b are the elution profiles for the 22° sodium chloride-soluble and -insoluble fractions, respectively. The columns (both 2.8 cm. i.d. x 25 cm.) were of DEAE-Sephadex and were developed identically. The columns were treated with the phosphate buffer from fractions 17-39. For both columns, 15 ml. fractions were collected at a rate of 0.3 ml./min. The fractions were assayed for uronic acid (●) and D-apiose (△) as described in the Materials and Methods.


0.2 M NaCl
0.25 M NaCl
0.3 M NaCl
(a)
Ia
Ib
IIa
IIb
III
15
10
5
0
3
2
1
0
0 40 55
75 95
120 130
155
E525 per ml of fraction (●)
E540 per ml of fraction (Δ)
Fraction number
(b)
I
II
IIIa
IIIb
15
10
5
0
3
2
1
0
0 40 55
75 95
120 130
155
E525 per ml of fraction (●)
E540 per ml of fraction (Δ)
Fraction number

Materials and Methods section. The analytical results are summarized in Table 3a.

The 22° sodium chloride-insoluble fraction (285.5 mg.) was dissolved in 40 ml. of the phosphate buffer and chromatographed on a DEAE-Sephadex column. The elution profile is given in Figure 1b. Additional washing with 0.5 N-sodium hydroxide did not result in the elution of more polysaccharide material. The indicated fractions were pooled and the polysaccharides were isolated. The analytical results are summarized in Table 3b.

The 70° sodium chloride-soluble fraction (190 mg.) was dissolved in 30 ml. of the phosphate buffer and chromatographed on a DEAE-Sephadex column. The elution profile is given in Figure 2a. Additional washing of this column with 0.5 N-sodium hydroxide did not result in the elution of more polysaccharide material. The indicated fractions were pooled and the polysaccharides were isolated. On a weight basis, the amount of material recovered as polysaccharides Ia, Ib, IIa, IIb and III was 9.8, 6.4, 28.8, 15.3, and 5.1%, respectively, of that applied to the column. Only polysaccharide IIa was analyzed. In the hydrogen form, it contained 34.1% D-apiose and 52.3% D-galacturonic acid and had $[\alpha]_{578}^{22}$ + 107.1°.

The 70° sodium chloride-insoluble fraction (91.0 mg.) was suspended in 40 ml. of water and stirred for several hours. Since all the material did not dissolve, the suspension was centrifuged. The supernatant solution was

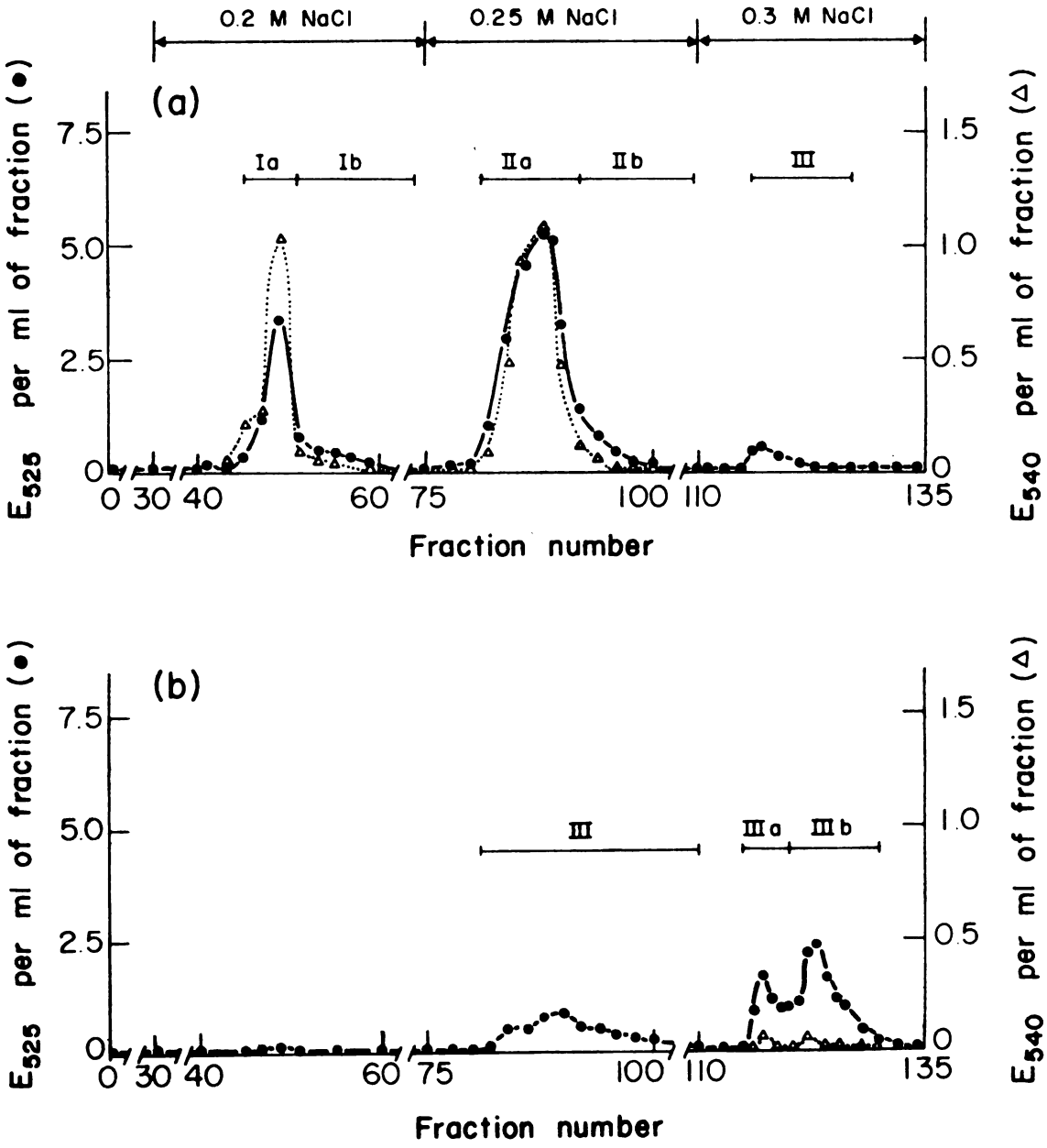
Table 3. DEAE-Sephadex chromatography of 22° sodium chloride-soluble and -insoluble fractions

The polysaccharides from the column were weighed as their sodium salts. They were converted in to the H^+ form as described in the text before characterization. The sugar values are percentages of the dried polysaccharides in their H^+ form expressed as glycosyl residues.

Polysaccharide	Amount recovered from column		D-Apiose	D-Galacturonic acid	$[\alpha]_{578}^{22}$
	(mg.)	(%)	(%)	(%)	
(a) 22° Sodium chloride-soluble fraction					
Ia	49.9	14.9	36.1	46.3	+91.8°
Ib	69.8	20.8	33.7	45.1	+99.5°
IIa	169.9	50.7	38.1	52.8	+106.9°
IIb	18.4	5.5	--	--	--
III	10.4	3.1	--	--	--
		95.0			
(b) 22° Sodium chloride-insoluble fraction					
I	10.0	3.5	--	--	
II	38.0	13.3	20.2	56.1	
IIIa	29.4	10.3	16.6	65.4	
IIIb	14.0	4.9	11.8	65.0	
		32.0			

Figures 2a and 2b. Column chromatograms of 70° sodium chloride-soluble and -insoluble fractions.

Figures 2a and 2b are the elution profiles for the 70° sodium chloride-soluble and -insoluble fractions, respectively. The columns (both 2.2 cm. i.d. x 25 cm.) were of DEAE-Sephadex and were developed identically. The columns were treated with the phosphate buffer from fractions 1-10 and with 0.1 M-sodium chloride in phosphate buffer from fractions 11-29. For both columns, 15 ml. fractions were collected at a rate of 0.25 ml./min. The fractions were assayed for uronic acid (●) and D-apiose (△) as described in the Materials and Methods.


0.2 M NaCl
0.25 M NaCl
0.3 M NaCl
(a)
Ia
Ib
IIa
IIb
III
7.5
5.0
2.5
0
1.5
1.0
0.5
0
0 30 40 60 75 100 110 135
Fraction number
E525 per ml of fraction (•)
E540 per ml of fraction (Δ)
(b)
III
IIIa
IIIb
7.5
5.0
2.5
0
1.5
1.0
0.5
0
0 30 40 60 75 100 110 135
Fraction number
E525 per ml of fraction (•)
E540 per ml of fraction (Δ)

decanted and the precipitate was dried with acetone and weighed (9.9 mg.). The material in the supernatant solution (81.1 mg.) was chromatographed on a DEAE-Sephadex column. The elution profile is given in Figure 2b. Additional washing of the column with 0.5 N-sodium hydroxide did not result in the elution of more polysaccharide material. The indicated fractions were pooled and the polysaccharides were isolated. On a weight basis, the amount of material recovered as polysaccharides II, IIIa, and IIIb was 20.6, 17.3, and 33.8%, respectively, of that applied to the column. No further work was done with these polysaccharides.

Fractionation of Sodium Chloride-Insoluble Fractions with Sodium Chloride

In addition to fractionation on a DEAE-Sephadex column, the 22° and 70° sodium chloride-insoluble fractions were also fractionated with sodium chloride.

The 22° sodium chloride-insoluble fraction from a different preparation (500 mg.) was dissolved in 100 ml. of water and 2.0 M-sodium chloride was added dropwise slowly to the constantly stirred solution until the desired molarities were reached. Precipitated polysaccharides were removed at 0.27 M-, 0.41 M- and 1.0 M-sodium chloride concentrations by centrifugation. Centrifugation was at 35000 x g. for 15 min. Increasing the sodium chloride concentration to 2.0 M by concentrating the supernatant solution from

the final centrifugation did not result in further precipitation. The precipitated polysaccharides were suspended in water and these suspensions and the supernatant solution were dialyzed until negative for chloride ion and then lyophilized. After desiccation, the polysaccharides were characterized. The results are summarized in Table 4a.

The 70° sodium chloride-insoluble fraction (200 mg.) was dissolved in 40 ml. of water and 2.0 M-sodium chloride was added as above. Precipitated polysaccharides were removed at 0.41 M- and 1.0 M-sodium chloride concentrations by centrifugation. Increasing the sodium chloride concentration to 2.0 M as described above did not result in further precipitation. The polysaccharides from the precipitated fractions and the supernatant solution were isolated as described above and characterized. The results are summarized in Table 4b.

Identification of Polysaccharide Components

The procedure used to determine the percent D-apiose content of the polysaccharide fractions in Table 1 also served to identify D-apiose as a component of these fractions. Solvents B and C distinguish between D-apiose, L-rhamnose, and L-fucose.

The identification of D-galacturonic acid as a component of the sodium chloride-soluble and -insoluble fractions was based on the following. All four fractions reacted positively in the sulfuric acid-carbazole test.

Table 4. Fractionation of 22° and 70° sodium chloride-insoluble fractions with sodium chloride

The polysaccharides were weighed and characterized as their sodium salts. The sugar values are percentages of the dried polysaccharides in the Na^+ form expressed as glycosyl residues. The polysaccharides were dissolved in distilled water for the optical-rotation measurements.

Polysaccharide	Concn. of NaCl	Type of precipitate	Amount recovered		D-Apiose	D-Galacturonic acid	$[\alpha]_{578}^{22}$
	(M)		(mg.)	(%)	(%)	(%)	
(a) 22° Sodium chloride-insoluble fraction							
A	0.19-0.27	Flocculent	29.6	5.9	7.9	77.5	+210.5°
B	0.27-0.41	Gel	335.0	67.0	9.2	80.4	+223.6°
C	0.73-1.00	Flocculent	59.2	11.8	17.2	62.3	+136.9°
D	Supernatant	---	39.5	7.9	24.7	59.2	+111.9°
				92.6			
(b) 70° Sodium chloride-insoluble fraction							
B	0.27-0.41	Gel	147.1	73.6	7.9	74.1	+192.1°
C	0.73-1.00	Flocculent	35.3	17.7	11.3	60.8	+178.8°
D	Supernatant	---	15.2	7.6	25.5	41.0	---
				98.9			

The insoluble fractions, and the soluble fractions after mild acid hydrolysis, were degraded in the presence of pectinase. Paper chromatography of the enzymatic hydrolysates of the insoluble fractions in solvent A revealed that D-galacturonic acid was the predominant sugar present. Finally, paper chromatography of representative polysaccharide fractions in solvents A and F following acid hydrolysis under reflux for 15 hr., showed that D-galacturonic acid was the major sugar present. These solvents distinguish between D-galacturonic and D-glucuronic acid. The D-galacturonic acid was further distinguished from D-glucuronic acid by its inability to form a lactone.

In the experiments for determining the D-apiose content of polysaccharide fractions we normally found a single spot on the chromatograms after chromatography with solvent B and it corresponded to D-apiose. Occasionally one and rarely two faint spots were present also. These were not identified. These results suggest that either other sugars are not present at all or only in small amounts in these polysaccharide fractions or there was incomplete hydrolysis.

Content of Esterified D-Galacturonic Acid Residues in Polysaccharide Fractions

The percent of esterified D-galacturonic acid residues in the 22° and 70° sodium chloride-soluble and -insoluble fractions was determined. The values for all

four fractions were similar, ranging from 1.0-3.5% with most values between 2.5 and 3.5%.

Partial Acid Hydrolysis of Polysaccharide IIa

The results of the partial acid hydrolysis and rechromatography on DEAE-Sephadex of 22° sodium chloride-soluble polysaccharide IIa (see Figure 1a) are shown in Figure 3. Paper chromatography in solvents A and B of aliquots of the column fractions containing reducing material revealed that D-apiose was the only sugar present. No reducing material was associated with the uronic acid-positive material. The uronic acid-positive material that was eluted corresponded to about 30% of theory and was characterized as a galacturonan by its lack of reactivity in the Nelson test and by its conversion to D-galacturonic acid on treatment with pectinase. No additional D-apiose was released on further acid hydrolysis of the eluted galacturonan.

Pectinase Hydrolysis of Polysaccharide Fractions

The results of the pectinase hydrolysis of the sodium chloride-soluble and -insoluble fractions are shown in Figures 4 and 5. From Figure 4 it can be seen that only the sodium chloride-insoluble fractions were extensively degraded by the pectinase treatment. Paper chromatography of the pectinase hydrolysates of these two fractions in solvent A revealed that the reducing material was predominantly D-galacturonic acid. The maximum reducing values

Figure 3. Column chromatogram of partially hydrolyzed polysaccharide IIa.

The 22° sodium chloride-soluble polysaccharide IIa (25 mg.) was hydrolyzed in 0.1 N hydrochloric acid for 30 min. at 100°. The solution was neutralized with sodium hydroxide and applied to a DEAE-Sephadex column (1.4 cm. i.d. x 20 cm.). The column was eluted first with 0.067 M-potassium dihydrogen phosphate-sodium hydrogen phosphate buffer, pH 7.7, and then with a step gradient of 0.1-0.3 M-sodium chloride in the same buffer. Fractions (6.0 ml.) were collected at a rate of 0.12 ml./min. and were assayed for uronic acid (●) and D-apiose (△) as described in the Materials and Methods.

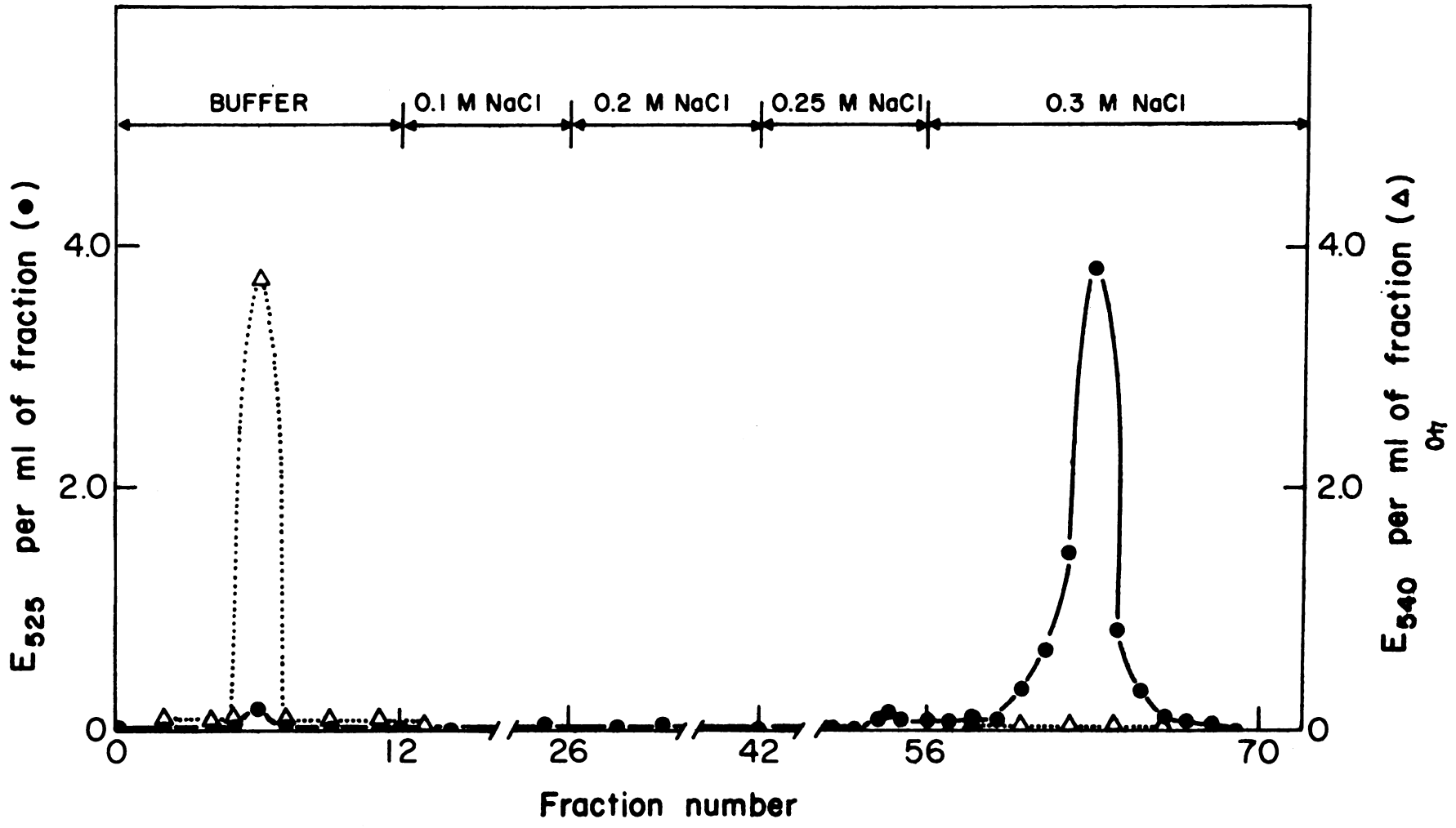

BUFFER
0.1 M NaCl
0.2 M NaCl
0.25 M NaCl
0.3 M NaCl
4.0
2.0
0
0
12
26
42
56
70
Fraction number
E525 per ml of fraction (●)
E540 per ml of fraction (△)
4.0
2.0
0

Figure 4. Pectinase hydrolysis of polysaccharide fractions.

Each point represents one assay mixture of 1.0 ml. final volume. Each assay mixture contained 500 µg. of the sodium salt of the polysaccharide fraction and 200 µg. of pectinase. Reaction termination was by addition of the Nelson reagents followed immediately by heating at 100°. In the zero time incubations, the order of addition of materials to the incubation tubes was the same as in the sample tubes except the Nelson alkaline copper reagent was added before the pectinase solution. Incubations were started such that all finished together. Reducing material was determined by the Nelson (1944) method. The polysaccharide fractions hydrolyzed were: (△), 22° sodium chloride-soluble fraction; (▲), 22° sodium chloride-insoluble fraction; (●), 70° sodium chloride-soluble fraction; (○), 70° sodium chloride-insoluble fraction.

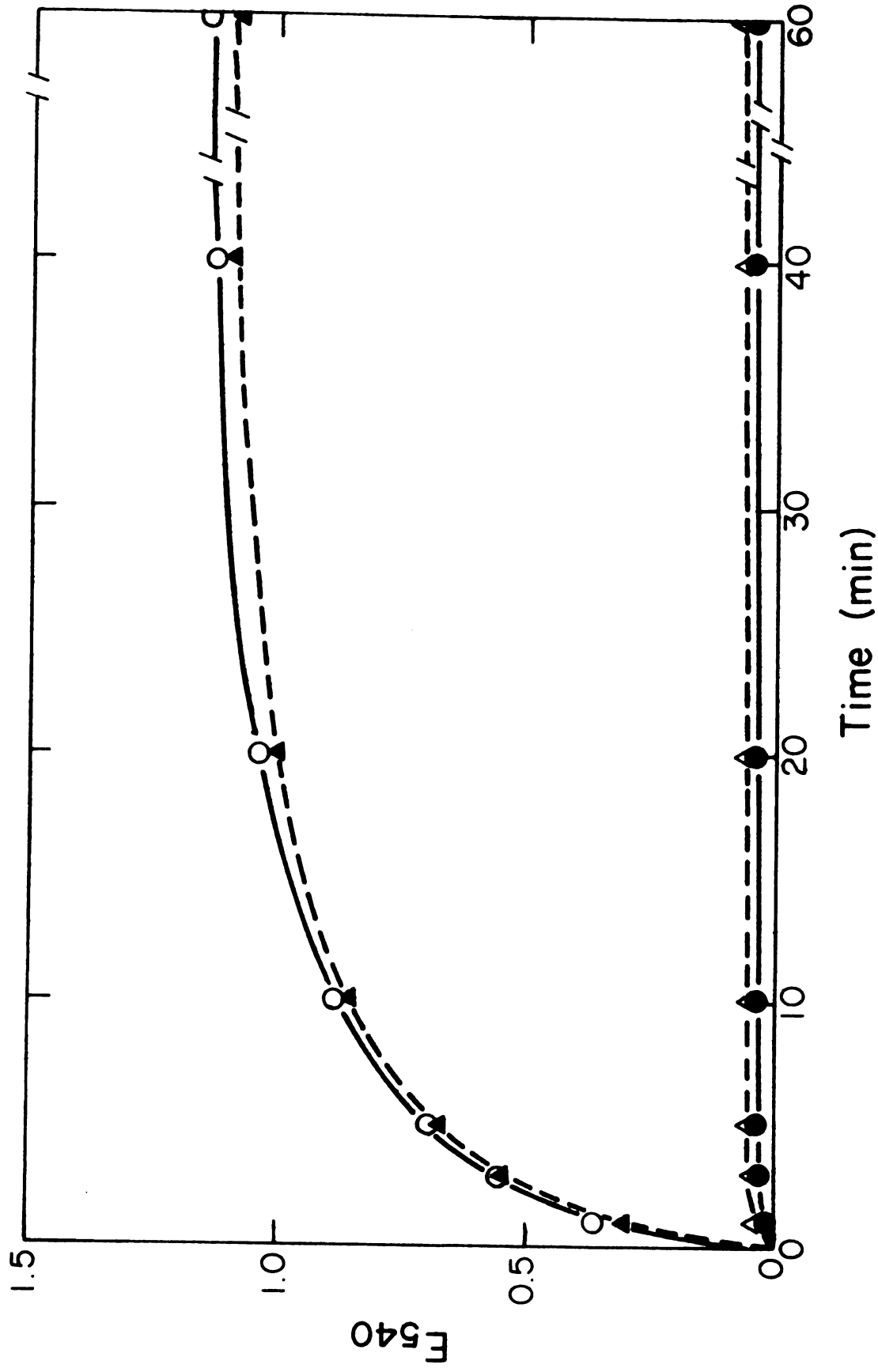
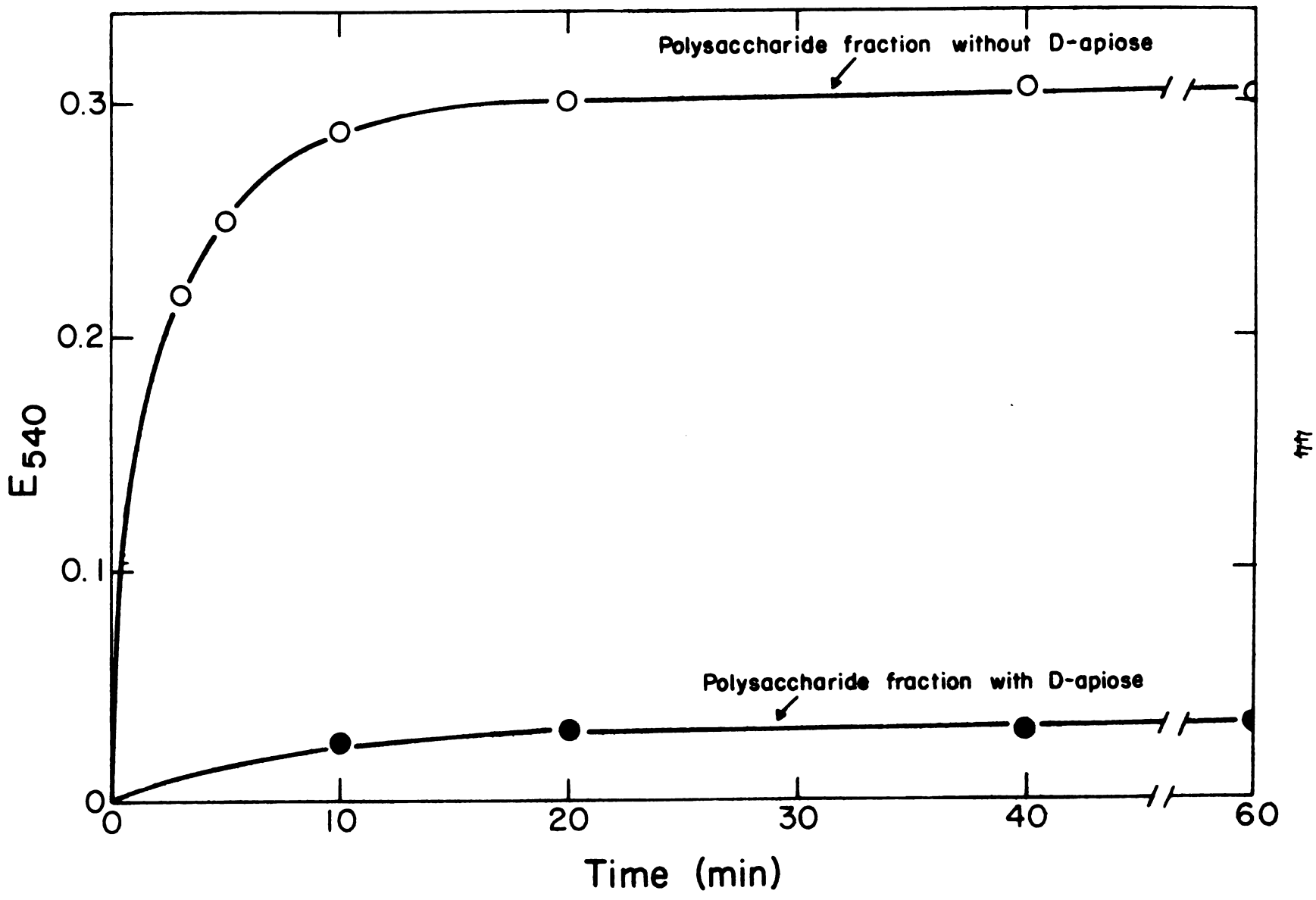

E540
1.5
1.0
0.5
0
0
10
20
30
40
60
Time (min)

Figure 5. Pectinase hydrolysis of the 22° sodium chloride-soluble fraction before (●) and after (○) removal of D-apiose.

Each point represents one assay mixture of 1.0 ml. final volume. Each assay mixture contained either 200 µg. of unhydrolyzed or 120 µg. of hydrolyzed polysaccharide fraction, both as the sodium salts, and 200 µg. of pectinase. The experimental procedure described in the legend of Figure 4 was followed. Reducing material was determined by the Nelson (1944) method.


Polysaccharide fraction without D-apiose
Polysaccharide fraction with D-apiose
E540
0.3
0.2
0.1
0
0
10
20
30
40
60
Time (min)

attained by the insoluble fractions correspond to a D-galacturonic acid release of about 80% of theory. The sodium chloride-soluble fractions were resistant to pectinase-catalyzed hydrolysis. However, incubation of such a polysaccharide fraction with pectinase after removal of the D-apiose by dilute acid hydrolysis resulted in extensive degradation, as shown in Figure 5. The maximum reducing value attained with the hydrolyzed 22° sodium chloride-soluble fraction corresponds to a D-galacturonic acid release of about 60% of theory.

Release of Formaldehyde Following Periodate Oxidation of Polysaccharide Material

The 22° sodium chloride-soluble and -insoluble fractions were oxidized with sodium metaperiodate and the amount of formaldehyde released was measured. In addition, certain of the polysaccharides obtained following separation of the 22° sodium chloride-soluble fraction on DEAE-Sephadex were also oxidized. The 22° sodium chloride-soluble polysaccharide IIa (see Figure 1a) was treated with sodium metaperiodate both before and after it was subjected to a hydrolysis procedure which liberated only D-apiose. These results are summarized in Table 5.

Table 5. Formaldehyde released on periodate oxidation of 22° extracted polysaccharides

Periodate oxidations were performed as described in the Materials and Methods. In the Table, 22° insoluble and 22° soluble refer to the 22° sodium chloride-soluble and -insoluble fractions, respectively. The 22° soluble Ia, Ib and IIa refer to the polysaccharides obtained from DEAE-Sephadex column chromatography of the 22° sodium chloride-soluble fraction (see Figure 1a). Hydrolyzed 22° soluble IIa was prepared by heating this polysaccharide for 30 min. at 100° in 0.5 ml. of 0.1 N-hydrochloric acid. The solution was cooled and an equal volume of 0.1 N-sodium hydroxide was added. Then 1.0 ml. of 0.05 M-sodium acetate buffer, pH 5.0, was added and the periodate oxidation was performed. The unhydrolyzed polysaccharide was dissolved in 1.0 ml. of 0.1 M-sodium chloride and an equal volume of 0.05 M-sodium acetate buffer, pH 5.0, was added. The theoretical yield of formaldehyde was calculated on the basis that one D-apiose residue yielded one molecule of formaldehyde.

Sample	D-Apiose	Formaldehyde expected	Formaldehyde found	Yield
	μmoles/mg. poly.*	μmoles/mg. poly.	μmoles/mg. poly.	% of theory
22° Insoluble	0.844	0.844	0.532	63.0
22° Soluble	2.590	2.590	1.428	55.1
Ia	2.735	2.735	1.571	57.5
Ib	2.550	2.550	1.301	51.2
IIa	2.880	2.880	1.518	52.8
22° Soluble IIa				
Unhydrolyzed	2.880	2.880	1.621	56.3
Hydrolyzed	2.880	2.880	2.845	98.8

*Poly. = polysaccharide

DISCUSSION

The procedure developed for the isolation of D-apiose-containing polysaccharides from the cell wall of L. minor is simple, mild and reproducible. The conditions used at 22° have no known degradative effect on these polysaccharides. The procedure is reasonably quantitative since 86% of the starting cell wall material and 97% of the D-apiose of the cell wall could be accounted for in the various fractions. With this procedure, the major apiogalacturonans can be obtained in sufficient quantity to permit structure determination. We established that the polysaccharides isolated were components of the cell wall by isolating cell walls before any fractionations were performed. An extensive series of extractions was used in the preparation of the cell walls to minimize possible contamination from other cellular components. Although this resulted in the solubilization of two-thirds of the plant material, 83% of the D-apiose was still present in the final product, designated cell walls. This is a minimum value since some of the cell wall material may have been solubilized by the shearing action of the blendor and some may have been lost in the cheesecloth during the filtrations. The cell walls were not further characterized to determine purity. The results clearly show that, at least in L. minor, most of the D-apiose present in this plant is in cell wall components. Its function there is unknown.

The polysaccharides isolated with our procedure belong to the pectic acid group of plant cell wall polysaccharides. The degree of esterification of the D-galacturonic acid in all polysaccharide fractions was very low. Similar results have been reported by Beck (1967). This appears to be their natural condition since the isolation procedure does not contain any steps which would lead to de-esterification. Highly esterified polysaccharides, if present in these cell walls, were probably lost during the preparation of the cell walls. Cold water-soluble polysaccharides, in general, would be lost during the isolation of cell walls by our procedure. Because of this, the weight value for the 70° water fraction in Table 1 is a minimum one.

The results in Table 1 show that on a dry weight basis, the four polysaccharide fractions extracted with ammonium oxalate made up 14% of the cell walls and contained 20% of the D-apiose originally present in these cell walls. They were presumably non-covalently bound in the cell wall, possibly as calcium salts, since they were readily extracted with ammonium oxalate. A portion of the apiogalacturonans could only be extracted by 70° ammonium oxalate. All of our experiments showed this material to be similar to that extracted at 22° but it may be partially degraded due to the temperature.

At 1.0 M-sodium chloride, the material extracted by

ammonium oxalate at 22° was separated into two fractions with strikingly different D-apiose contents, 33.1% versus 10.1%. Similar, though not so striking, results were obtained with the material extracted at 70°. Although the 22° sodium chloride-soluble fraction separated on DEAE-Sephadex into two or possibly three polysaccharides, the differences between these polysaccharides were slight and may represent natural variations within one type of polysaccharide or possibly some degradation of one type of polysaccharide. The DEAE-Sephadex chromatography of the 22° sodium chloride-insoluble fraction was not entirely successful. The yields were low and the resolution was incomplete. However, sufficient information was obtained to show that it was a mixture of partially separable components. The chemical and physical properties of the polysaccharides obtained from the 22° sodium chloride-insoluble fraction are different from those of the 22° sodium chloride-soluble fraction. The elution profiles of these two fractions show that polysaccharide II of each fraction is eluted in about the same position. Since the results in Table 3 show that these two polysaccharides are different, it is clear that in order to obtain pure 22° sodium chloride-soluble polysaccharide II, the 1.0 M-sodium chloride fraction step is essential.

The 22° and 70° sodium chloride-insoluble fractions were resolved into four and three components, respectively,

by fractionation with sodium chloride. In both cases, the recovery of material was virtually quantitative in contrast with the recoveries from the DEAE-Sephadex columns. The polysaccharides of both insoluble fractions were precipitated at similar and definite sodium chloride molarities. The three polysaccharides of the 70° insoluble fraction have properties similar to polysaccharides B, C and D of the 22° insoluble fraction, again showing that the material extracted at each temperature is very similar. These experiments and those discussed above show that sodium chloride can be used successfully to fractionate the apiogalacturonans. They confirm that the two insoluble fractions are mixtures of polysaccharides. Fractionation of pectic substances from plant material with inorganic univalent cations has been previously used successfully by Bhattacharjee and Timell (1965) and Zitko and Bishop (1965).

The results in Table 4 show that there is a direct relationship between the D-apiose content of a polysaccharide and its solubility in sodium chloride solutions; the higher the D-apiose content, the greater the solubility. The apiogalacturonans which contained the greatest percentage of D-apiose were soluble even in 2 M-sodium chloride. However, they were readily precipitated by low concentrations of the bivalent calcium ion. In view of the very low D-galacturonic acid methyl ester content of all of the apiogalacturonans isolated, the sodium chloride fractionations

reported here are probably based on the ability of D-apiose to interfere with the formation of ionic bonds between the negative charges of the apiogalacturonan and the positive sodium ions. If precipitation occurred by the formation of ionic bonds between polysaccharide molecules via the sodium and chloride ions and if the D-apiose sterically shielded the negative charges on the polysaccharide molecules, those polysaccharides of highest D-apiose content would have the greatest difficulty to form such bonds and thus to aggregate. Consequently, these polysaccharides would be the most soluble in salt solutions.

From Figure 3 it can be seen that hydrolysis of the 22° sodium chloride-soluble polysaccharide II with dilute acid resulted in the removal of the D-apiose from the polysaccharide. The remainder of the molecule appeared to be a galacturonan as evidenced by its quantitative conversion to D-galacturonic acid by pectinase treatment. The galacturonan was relatively undegraded by the acid hydrolysis treatment as evidenced by its elution as a single peak from DEAE-Sephadex. However, only 30% of the expected uronic acid-positive material was eluted from the DEAE-Sephadex column.

The pectinase hydrolysis results depicted in Figure 4 suggest that in the sodium chloride-soluble polysaccharides, the D-apiose is attached to the galacturonan throughout its length since these polysaccharides are resistant to pectinase-catalyzed hydrolysis. This is substantiated by the results

in Figure 5 which show that these polysaccharides are readily hydrolyzed by treatment with pectinase if their D-apiose is removed. Since there is very little methyl esterification, the inhibition must have been due to the D-apiose side-chains. If the D-apiose was concentrated at a few specific points in these polysaccharides, a large proportion of the D-galacturonic acid should have been released by pectinase treatment before acid hydrolysis. Since this was not the case, such a structure can be excluded. In the case of the sodium chloride-insoluble polysaccharides, their relatively small D-apiose content dictates that large portions of the galacturonan backbone must be free of D-apiose side-chains, thus making them susceptible to pectinase. In these polysaccharides, two types of structures are possible. Either the D-apiose is attached throughout the length of the galacturonan with sufficient space in between the D-apiose molecules to allow the pectinase to act or all the D-apiose molecules are concentrated in one portion of the galacturonan leaving the remainder susceptible to pectinase. The available data do not allow us to make a choice. Because the apiogalacturonans from L. minor were degraded by the pectinase, we have assumed that they consist of α-(1→4)-linked D-galacturonic acid residues. The two apiogalacturonans isolated by Beck (1967) were both extensively degraded by pectinase.

The ability of D-apiose to confer resistance to pectinase on apiogalacturonans of high D-apiose content has not been observed before and could be physiologically significant. Recent studies have shown that an early consequence of pathogen infection in plants is the degradation of cell wall polysaccharides (Bateman, Van Etten, English, Nevins and Albersheim, 1969; English and Albersheim, 1969). Thus, one function of D-apiose in L. minor might be to protect the pectic substances from degradation by infecting pathogens. If D-apiose functions in this manner in L. minor, it probably functions similarly in other plants that contain D-apiose. It will be of interest to survey such plants for apiogalacturonans. Pertinent to this is the isolation, by Ovodova et al. (1968), of a D-apiose-containing polysaccharide from three species of the Zosteraceae family which also contained D-galacturonic acid. D-Apiose-containing polysaccharides have only been isolated from L. minor and the three species of the Zosteraceae family. In an early study on D-apiose, Bell et al. (1954) suggested that the resistance of P. australis and Z. marina fibers to natural decomposition may be due to the presence of a D-apiose derivative.

The acid and enzymatic hydrolysis data indicate that the apiogalacturonans isolated consist of an unesterified galacturonan backbone to which are attached side-chains of D-apiose. Polysaccharides similar to those described here

have been isolated from plant material by Aspinall and Baillie (1963) and by Bouveng (1965). However, in these polysaccharides the side chains consist of sugars other than D-apiose. This type of polysaccharide where sugars are attached as side chains to a galacturonan backbone may be widely distributed in plants.

Beck's acid hydrolysis and autohydrolysis data (1967) and his inability to detect disaccharides or higher homologues of D-apiose, led him to suggest that the D-apiose was attached to the galacturonans as monomeric side-chains. However, our formaldehyde data indicated that the D-apiose was partially substituted since only about 50% of the theoretical quantity of formaldehyde was obtained on periodate oxidation. Only D-apiose will yield formaldehyde in these polysaccharides, and it would have to be substituted at position 3 or 3' to block formaldehyde production during periodate oxidation. The theoretical amount of formaldehyde (i.e., 1 mole/mole D-apiose) was obtained if the apiogalacturonan was first hydrolyzed under conditions which resulted in the release of only free D-apiose. The most plausible explanation of the findings is to postulate the existence of acid-labile disaccharide side-chains of D-apiose attached to a galacturonan. Recently we have isolated a disaccharide of D-apiose from several apiogalacturonans by heating an aqueous 0.3% (w/v) solution of the apiogalacturonan at 100° for 3 hr. at pH 4.5 (Part III).

The data in Table 1 show that on a dry weight basis, 76% of the D-apiose of the cell wall of L. minor was not solubilized by our extraction procedure. Of interest will be the determination of the structure of D-apiose-containing polysaccharides remaining in the residue. If D-apiose is functioning to protect the pectic substances, the D-apiose remaining in the residue may be functioning similarly but protecting different polysaccharides, in which case different D-apiose-containing polysaccharides would be present. The isolation by Williams and Jones (1964) of a crude polysaccharide fraction containing D-xylose and D-apiose in the approximate ratio of 1:1 from the marine plant, Z. marina, showed that other types of D-apiose-containing polysaccharides are present in nature. Alternatively, the D-apiose could be present in polysaccharides that are the same as those reported here but for unknown reasons are not extracted. A major obstacle to further extraction of the L. minor cell wall is in developing sufficiently mild procedures for solubilizing the D-apiose-containing polysaccharides still in the residue. One possible approach is to use a purified enzyme to selectively degrade one type of polysaccharide (e.g., cellulose) and determine if the remaining polysaccharides are now more readily extractable.

PART 3

ISOLATION AND CHARACTERIZATION OF APIBIOSE FROM APIOGALACTURONANS

INTRODUCTION

The occurrance of D-apiose (3-C-hydroxymethyl-aldehydo-D-glycero-tetrose) as a component of several different types of compounds, isolated from a large number of plants, was briefly reviewed in Part II. In the same article, we reported the isolation of a series of apiogalacturonans from L. minor. The evidence presented indicated that the D-apiose was glycosidically linked as sidechains on galacturonans. Contrary to the conclusion of Beck (1967), the evidence also indicated that the sidechains were disaccharide units of D-apiose rather than monosaccharide units. Periodate oxidation of the intact apiogalacturonans indicated that the glycosidic linkage between the two D-apiose molecules of a sidechain was either 1→3 or 1→3'.

Further investigations on the structure of the apiogalacturonans from L. minor, has led to the isolation of a disaccharide of D-apiose. We give the common name apibiose to this disaccharide. Apibiose is released from the apiogalacturonans by a very mild reaction which apparently is a hydrolysis. We report here the structural determination of apibiose and several parameters of the reaction involved in its release from the apiogalacturonans.

MATERIALS AND METHODS

Materials

Apiin and D-apiose were obtained as described in Part II. Barium [^{14}C] carbonate was obtained from New England Nuclear Corp. and was converted to sodium [^{14}C] bicarbonate (5 μCi/μmole) before use. Fungal pectinase was purchased from Sigma Chemical Company. The enzyme was purified 30-fold over the crude preparation by the manufacturer who stated it still contained several other enzymes. The specific activity of this batch (Lot 125B-0350) as measured by Sigmna was 0.7 units per mg. of solid. A unit was defined as that amount of enzyme which liberated 1 μmole of D-galacturonic acid per min. at 25° and at pH 4.0 with de-esterified citrus pectin as the substrate.

Plant Material

L. minor was grown on modified medium V of Norris, Norris and Calvin, 1954, as described by Kindel (manuscript in preparation). Large quantities of L. minor were obtained from the Battle Creek River at Bellvue, Michigan. The plant material obtained from the river was washed extensively and either used immediately or stored at -20°.

General Methods

D-Apiitol was prepared from D-apiose by catalytic hydrogenation (Neal and Kindel, submitted to J. Bacteriol.). D-Apiose phenylosotriazole was prepared by the method of

Kindel (1969) and D-apiose α-benzyl-α-phenylhydrazone was prepared as described by Schmidt (1930). Solutions were concentrated under reduced pressure by rotatory evaporation at temperatures less than 35°. Melting points were determined with a Kofler micro hot stage (A. H. Thomas Co.) and are uncorrected. Optical rotations were determined with a Zeiss Photoelectric Precision Polarimeter 0.005° (Carl Zeiss, Oberkochen, Wuerttemberg, Germany) at 22° and at 578 mμ and 546 mμ. Molecular rotations were calculated from specific rotations by the following formula:

$$[M] = \frac{[\alpha] \times M.W.}{100}$$

Infrared spectra were obtained with a Perkin-Elmer grating infrared spectrophotometer, model 337, with air as the reference. Samples were prepared as potassium bromide pellets or as smears on potassium bromide pellets. Proton magnetic resonance spectra were obtained at 60 MHz and 100 MHz, with Varian A60 and HA100 spectrometers, respectively. Samples were dissolved in deuterium oxide and exchangeable hydrogens were removed by concentration of the sample several times from deuterium oxide. The final concentration of the sample for spectral analysis was 10% (w/v). The spectra were obtained at ambient temperatures with tetramethylsilane as the external standard ($\tau = 10$). Mass spectra were obtained by the direct probe method with a LKB mass spectrometer, model 9000 (LKB Instruments, Inc.,

Stockholm, Sweden). Spectra were recorded at 70 ev., with an accelerating voltage of 3.5 kv., an ion source temperature of 210°, and a filament current of 60 μa. Radioactivity was detected on chromatograms with a Packard strip counter, model 7201. All other radioactivity measurements were made with a Packard Tri-Carb liquid scintillation counter, model 3310, employing one of the following scintillation solutions: A) Bray (1960), or B) 2,5-bis-[2(5-tert-butylbenzoxazolyl)]-thiophene-toluene (4 g./l.).

Chromatography

Descending paper chromatography was used and was usually carried out with Whatman No. 3MM paper. This paper was prewashed with 0.1 M citric acid followed by distilled water unless otherwise noted. Unwashed Whatman No. 1 paper was used for methylated sugars. The following solvents were employed: A) ethyl acetate-acetic acid-formic acid-water (18:3:1:4, by vol.), B) propan-2-ol-water (9:1, by vol.), C) propan-1-ol-ethyl acetate-water (7:1:2, by vol.), D) pyridine-ethyl acetate-acetic acid-water (5:5:1:3, by vol.), E) butan-1-ol-pyridine-benzene-water (5:3:1:3, by vol., upper phase), F) water saturated 2-butanone (redistilled), G) butan-1-ol-ethanol-water (10:3:3, by vol., upper phase). Sugars were detected on chromatograms with aniline hydrogen phthatlate (Partridge, 1949) or by the silver nitrate dip method (Trevelyan, Procter, and Harrison, 1950).

Gas-liquid chromatography was carried out with a F and M Corp. gas chromatograph, model 402, equipped with a hydrogen flame detector. The 6 ft. x 1/8 in. i.d. U-shaped borosilicate glass column was packed with acid washed and dimethyldichlorosilane treated Chromasorb W coated with 3% OV-1 (Applied Science Laboratories, State College, Pa.) and was used at 110°. The internal standard was 2,3,4,6-tetra-O-methyl-D-glucose.

Radioactive L. minor

Radioactive L. minor were obtained by exposing a small number of fronds in a closed chamber to increasing amounts of [^{14}C] carbon dioxide over a 30 day period. The plants were grown under continuous incandescent light (approx. 100 ft-candles) at 22-24° on modified medium V. In the 30 day period, the number of fronds doubled every 3 days. The radioactive carbon dioxide was administered every fourth day in amounts which varied from 5 μCi. at the beginning of the experiment to 100 μCi. at the end. The chamber was kept closed for 24 hr. after each administration of [^{14}C] carbon dioxide and was then opened to the atmosphere for the next 48 hr. The total radioactive carbon dioxide administered was 415 μCi. The labelled plant material was diluted 5-fold with unlabelled L. minor, grown under similar conditions, before use.

Isolation of Apiogalacturonans

Apiogalacturonans were isolated as the sodium salts as described in Part II. [^{14}C] Apiogalacturonans were isolated from radioactive L. minor by the same procedure and had a specific activity which varied from 14000 disintegrations min.$^{-1}$ mg.$^{-1}$ apiogalacturonan to 1800 disintegrations min.$^{-1}$ mg.$^{-1}$ apiogalacturonan.

Degradation of [^{14}C] Apiogalacturonans

[^{14}C] Apiogalacturonans were degraded in screw-top test tubes (13 mm. o.d. x 100 mm.) as described in the individual experiments reported. The solutions were then spotted directly on unwashed Whatman No. 3MM paper and developed in solvent A. The dried chromatograms were scanned for radioactivity and the labelled areas were cut out and counted directly in scintillation solution B at 60% efficiency. The chromatograms all contained only the three labelled areas shown in Figure 1a. Greater than 90% of the starting radioactivity could be accounted for in these labelled areas.

Isolation of Apibiose

Either radioactive or non-radioactive sodium apiogalacturonates were dissolved in water to yield a 1% (w/v) solution. The pH of the solution was 4.3-4.5. The solution was heated for 3 hr. at 100°, cooled and concentrated to approximately a 3% (w/v) solution. Three volumes of

acetone was poured slowly into the stirred solution. The resulting suspension was placed at 4° for 4 hr. and then centrifuged at 15000 x g. for 20 min. The supernatant solution was decanted and the precipitate was resuspended in 1 volume of acetone. The precipitate was removed by recentrifugation at 15000 x g. for 20 min. The combined supernatant solutions were concentrated to a small volume and an aliquot was chromatographed in solvent A. The precipitate was dissolved in water to yield a 1% (w/v) solution and the above described treatment was repeated until there was only a small amount of apibiose present in the supernatant fraction. For the radioactive apiogalacturonans a single heat treatment was sufficient. However, in the larger scale non-radioactive isolations, 3-6 heat treatments were carried out.

Radioactive apibiose was purified by preparative paper chromatography. The supernatant solution from the above described process was streaked on Whatman No. 3MM paper and the chromatograms were developed in solvent A. The dried chromatograms were scanned for radioactivity and the apibiose area was cut out and eluted with water. The eluate was concentrated to a small volume. The yield of radioactive apibiose was determined by counting an aliquot of the radioactive solution in scintillation solution A.

The non-radioactive apibiose was purified by partition column chromatography on powdered cellulose. The

combined supernatant solutions from the above described process were concentrated to a small volume. Whatman powdered cellulose (W. and R. Balston, Ltd., England, standard grade) was added to the solution until it became a thick suspension. The suspension was dried over phosphorus pentoxide *in vacuo*. The cellulose column was prepared by a modification of the procedure described by Whistler and BeMiller (1962). The cellulose was suspended in acetone by stirring and the fines were removed by decanting after standing for 5 min. The process was repeated twice. The cellulose was resuspended in acetone by stirring and then poured into a glass column (5 cm. i.d. x 80 cm.) which contained a plug of glass wool on the bottom and acetone to a depth of approximately 20 cm. The column was completely filled with the slurry of cellulose and then the stopcock at the bottom of the column was opened. The column was kept completely filled with the slurry until the desired amount was added. The column of cellulose (5 cm. i.d. x 40 cm.) was washed with 1-2 liters of acetone. The acetone was drained to the top of the column and then water saturated butan-1-ol was added. The column was equilibrated with 6 liters of water saturated butan-1-ol. The solvent was then drained to 1-2 cm. above the top of the cellulose and a circle of Whatman No. 5 paper was placed on top of the cellulose. The sample was applied to the top in the dry state. A few ml. of water

saturated butan-1-ol was then added to facilitate removal of any trapped air. Another circle of Whatman No. 5 paper was placed on top of the sample. Solvent was added and a reservoir was attached. The column was then developed at a rate of 1 ml./min. with 10 ml. fractions being collected. Aliquots of the fractions were spotted on paper. The paper was developed by the silver nitrate dip method. The fractions containing the apibiose were pooled and concentrated to a syrup. The syrup was dissolved in water and treated with acid washed activated carbon (Darco G-60, Atlas Chemical Industries, Inc.). The charcoal was removed by filtration and the filtrate was passed through a Seitz filter. The filtrate was concentrated to a syrup and dried over phosphorus pentoxide in vacuo. The yield of chromatographically pure apibiose, $[\alpha]_{578}^{22}$-69.1° (C5, water), calculated on the D-apiose content of the starting apiogalacturonans, was dependent on the number of times the degradation procedure was repeated.

The cellulose column could be reused several times without alteration of the elution profile of the chromatogram if the cellulose on which the sample was adsorbed was removed each time.

Sodium Borohydride Reduction of [^{14}C] Apibiose (peak 2)

Radioactive apibiose was reduced with a 10-fold excess of sodium borohydride in water at 37° for 16 hr.

The solution was neutralized with acetic acid and then concentrated to dryness. The borate was removed as the methyl-ester by distillation under reduced pressure. The reduced material was purified by paper chromatography on Whatman No. 3MM paper, first in solvent A and then in solvent D. The material migrated as a single peak in the latter solvent.

Hydrolysis of [^{14}C] Apibiose and Reduced [^{14}C] Apibiose (peak 2)

Before radioactive apibiose and reduced apibiose were hydrolyzed, unlabelled D-apiose was added to the former and unlabelled D-apiose and D-apiitol were added to the latter. Both compounds were hydrolyzed with 0.1 N-sulfuric acid at 100° for 1 hr. The solutions were cooled and neutralized with sodium hydroxide to pH 6. The solutions were spotted directly on Whatman No. 3MM paper and the chromatograms were developed in solvent A. The chromatograms were scanned for radioactivity and the labelled areas were cut out and counted in scintillation solution B.

Preparation of Apibiose Phenylosotriazole

Apibiose was dissolved in sufficient 5.0 M-sodium acetate buffer, pH 4.5, to yield a 2% (w/v) solution which was then heated to 80°. A 10-fold molar excess of phenylhydrazine hydrochloride in an equal volume of the same buffer was heated to 80°. The solutions were mixed and heated for 1 hr. at 90-95°. Three volumes of water at 22° was added to

the hot solution and the resulting precipitate was allowed to settle at 4° for 2-3 hr. The precipitate was collected by centrifugation at 35000 x g. for 20 min. The precipitate was dissolved in 1-3 ml. ethanol and 10 volumes of water was immediately added. After cooling to 4° for 4 hr., the resulting precipitate was collected by centrifugation and dried over phosphorus pentoxide in vacuo. The yield of amorphous apibiose phenylosazone, m.p. 91-93°, was 75-80%. (Found: C,55.7; H,5.9; N,11.8; $C_{22}H_{27}N_4O_7$ requires C,57.5; H,5.9; N,12.2%).

The apibiose phenylosazone was suspended in water to yield a 1.5% (w/v) suspension which was then heated to reflux. A solution of 1.1 molar equivalents of cupric sulfate pentahydrate in 0.5 volumes of water at 80° was added to the phenylosazone suspension and the resulting reddish solution was refluxed for 1 hr. The solution was cooled to 4° and then filtered through Whatman No. 5 paper. The filtrate was concentrated to a small volume and then streaked on several sheets of unwashed Whatman No. 3MM paper. The papers were developed in solvent A. The fluorescing bands at R_F 0.69, detected by 366 mμ ultraviolet light, were cut out and eluted with water. The eluate was concentrated to 5-10 ml. and then continuously extracted with diethyl ether for 30-36 hr. The diethyl ether solution was treated with Darco G-60 and then filtered. The filtrate was concentrated with a stream of air

until the solution became cloudy. Crystallization occurred on standing at 22°. The resulting colorless needle-like crystals, m.p. 116.5-117.0° and $[\alpha]^{22}_{578}$-80.0° (C5, water) were obtained in a yield of 25% from apibiose phenylosazone, for an overall yield of 20%. The apibiose phenylosotriazole was recrystallized from diethyl ether by solution in water and re-extraction with diethyl ether. (Found: C,51.9; H,5.8; N,11.4; $C_{16}H_{21}N_3O_7$ requires C,52.3; H,5.8; N,11.5%).

Periodate Oxidation and Determination of Formaldehyde

Samples of apibiose and apibiose phenylosotraizole (4 to 17 mg.) were dissolved in 1 to 2 ml. of 0.05 M-sodium acetate buffer, pH 5.0, and then a 2.5-fold theoretical excess of sodium metaperiodate, in an equal volume of water, was added. The solutions were kept at 22° for 2 hr. in the dark. Preliminary experiments had shown that formaldehyde production was complete after 1 hr. The solutions were passed through a column (1 cm. i.d. x 7 cm.) of Dowex 1-X8 (200-400 mesh, acetate form). The formaldehyde quantitatively passed through the column and was determined with chromotropic acid (recrystallized once from aqueous 50% (v/v) ethanol).

Methylation Analysis

The methylation procedure was essentially that of Hakamori (1964). However, the methylsulfinyl anion was

generated as described by Sandford and Conrad (1966). The methylated compounds were extracted from the reaction mixture with chloroform. The extent of methylation, as a function of hydroxyl absorption, was determined by infrared spectroscopy. Methylated compounds were hydrolyzed with 1 N-sulfuric acid for 1 hr. at 100°. The hydrolysates were neutralized with sodium hydroxide to pH 6 and then 9 volumes of ethanol were added. The insoluble sodium sulfate was removed by centrifugation and the supernatant solutions were decanted. The supernatant solutions were concentrated for chromatography.

Standard 2,3,3'-tri-O-methyl-D-apio-D-furanose (Halyalker, Jones and Perry, 1965) was isolated from the supernatant solution of a hydrolysate of 1 g. of methylated apiin by partition chromatography on a column (2 cm. i.d. x 41 cm.) of powdered Whatman cellulose. The column was poured and equilibrated with water saturated redistilled 2-butanone (Sandford and Conrad, 1966). The sample was prepared and applied to the column as described for the isolation of non-radioactive apibiose. The column was then developed with the same solvent. Fractions (3 ml./14 min.) were collected and aliquots were chromatographed on Whatman No. 1 paper in the same solvent. The sugars were visualized with aniline hydrogen phthalate. The fractions containing chromatographically pure 2,3,3'-tri-O-methyl-D-apio-D-furanose were pooled and concentrated to a small volume.

Standard 2,3,4,6-tetra-O-methyl-D-glucose was prepared from D-glucose by this procedure also.

Isolation and Pectinase Hydrolysis of Partially Degraded [^{14}C] Apiogalacturonans

The precipitate obtained on addition of acetone to the thermally degraded [^{14}C] apiogalacturonans (see above section on Isolation of Apibiose) was designated partially degraded [^{14}C] apiogalacturonans. This precipitate was dissolved in water, concentrated to a small volume and freeze-dried.

One (1.0) mg. of partially degraded [^{14}C] apiogalacturonans were hydrolyzed with 2 mg. of pectinase for 2 hr. by the procedure described in Part 2. The hydrolysis was terminated by heating for 1 min. at 100°. The denatured protein was removed by centrifugation. The supernatant solution was spotted directly on unwashed Whatman No. 3MM paper and developed in solvent D. The chromatograms were dried, scanned for radioactivity and the labelled areas were cut out and counted in scintillation solution B. Percent hydrolysis was the percent of the total recovered disintegrations/min. in D-galacturonic acid.

RESULTS

Degradation of [^{14}C] Apiogalacturonans

When sodium [^{14}C] apiogalacturonates isolated from *L. minor* were heated in water at 100° and then chromatographed

in solvent A, two radioactive peaks of high R_F were detected (Figure 1). In the unheated control all of the radioactivity remained at the origin of the chromatogram. The same two peaks were obtained from all four apiogalacturonan fractions obtained by ammonium oxalate extraction (Table 1, Part 2). However, treatment of the 70° water fraction and the residue fraction in a similar manner did not yield these two compounds. Because of the high D-apiose content of the 22° sodium chloride soluble IIa (22SCS-IIa) apiogalacturonan (Table 3, Part 2), this polysaccharide was used primarily for the characterization of the degradation reaction, where degradation is defined as the percent of the total recovered disintegrations/min. in peaks 1 and 2.

This degradation reaction was dependent on the temperature and the length of the heating period (Figure 2). Degradation at 100° occurred very rapidly and reached a final value of 40% degradation after 5 hr. There was still extensive degradation at 80°. However, the degradation after 7 hr. at 60° was only 10% of that which occurred at 100°.

The degradation reaction was also dependent on the pH of the solution (Figures 3 and 4). The rate of the degradation declined steadily from pH 3.5 to 6.0 until it dropped to essentially zero at pH 6.5. There was also a change in the ratio of the two compounds obtained during

Figure 1. Scan of a paper chromatogram of thermally degraded sodium [^{14}C] apiogalacturonate from L. minor.

Aliquots (0.1 ml.) of a solution of apiogalacturonan in water (3 mg./ml.) at pH 4.5 were heated at 100° for 3 hr. (a) or kept at 22° (b). The assays were treated as described in the Materials and Methods.

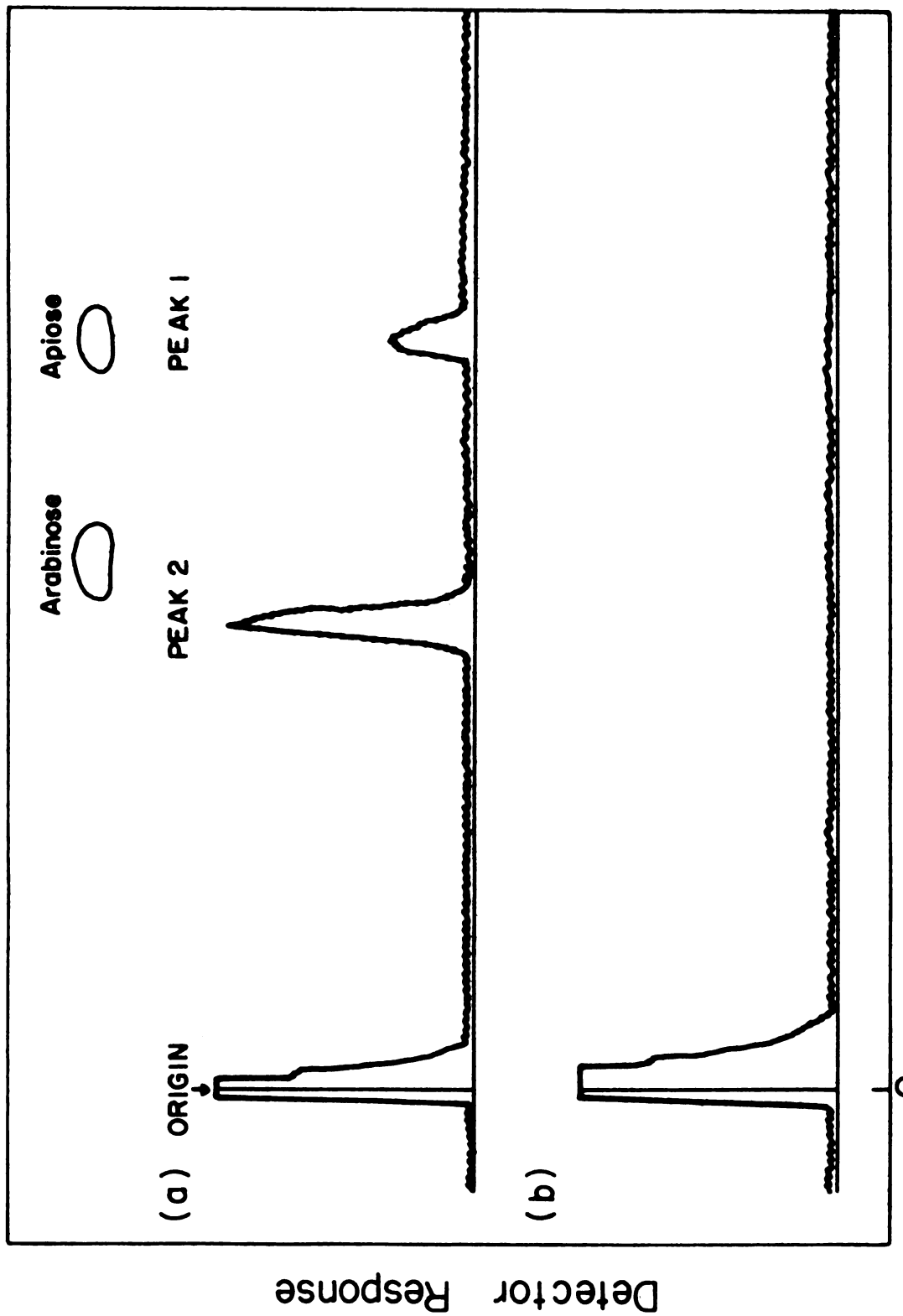

Detector Response
(a)
ORIGIN
PEAK 2
Arabinose
PEAK 1
Apiose
(b)
0
Centimeters

Figure 2. Degradation of [^{14}C] apiogalacturonan 22SCS-IIa as a function of time and temperature.

The polysaccharide, as the sodium salt, was dissolved in water at a concentration of 3 mg./ml. The pH of the solution was 4.5. Aliquots of 0.1 ml. were used for each assay. The assays were heated for 1, 3, 5, and 7 hr. at 100° (●), 80° (○), and 60° (▲) and were started in such a way that all were completed at the same time. The assays were treated as described in the Materials and Methods. Percent degradation is the percent of the total recovered disintegrations/min. in peaks 1 and 2.

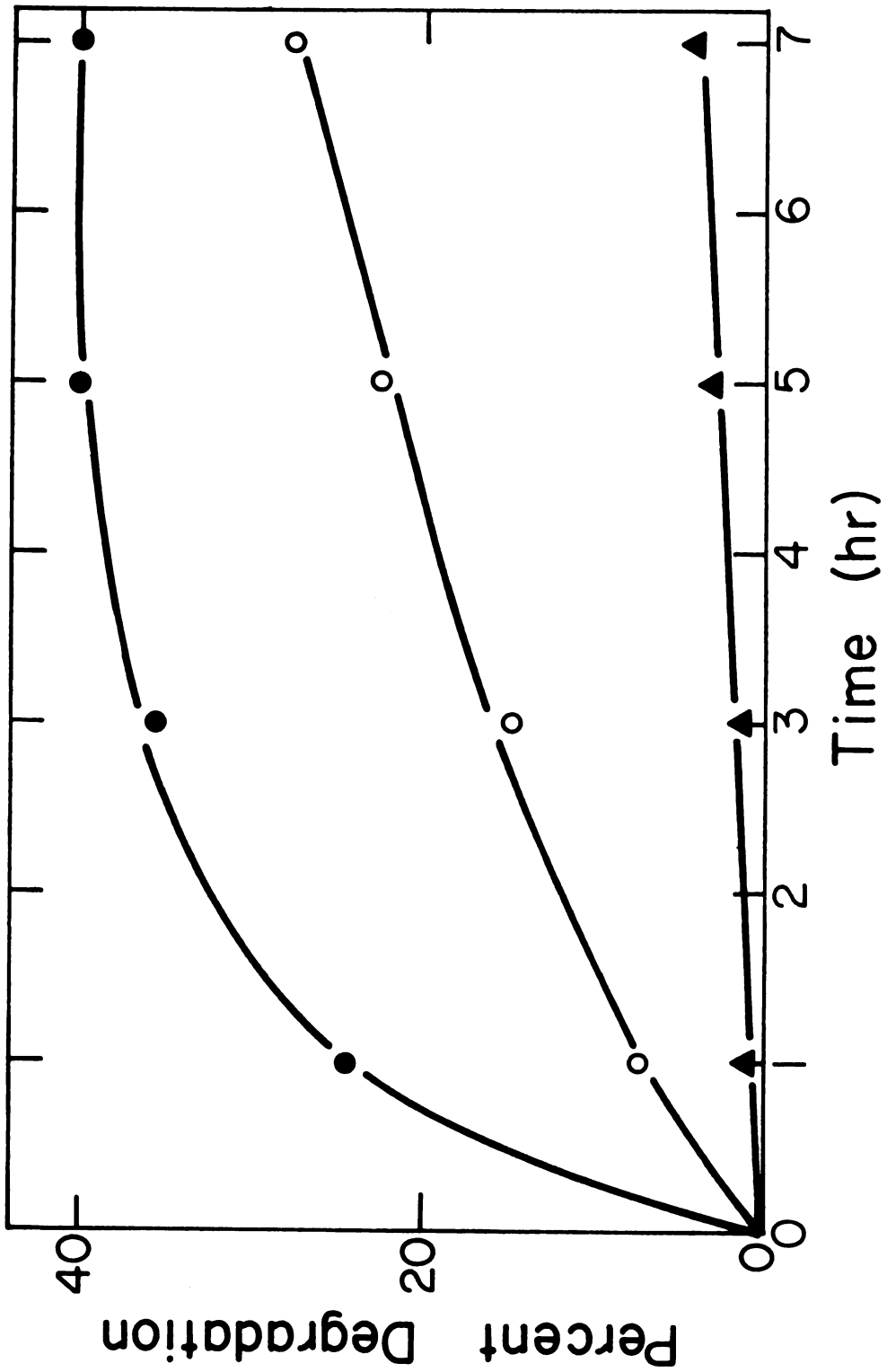

Percent Degradation
0
20
40
0
1
2
3
4
5
6
7
Time (hr)

Figure 3. The rate of degradation of [^{14}C] apiogalacturonan 22SCS-IIa as a function of pH.

The sodium salt of the polysaccharide was dissolved in 0.05 M-potassium phosphate buffer, pH 3.5 (▲), 4.5 (●), 5.5 (△), 6.0 (■), 6.5 (○), and 8.0 (□), at a concentration of 3 mg./ml. Aliquots of 0.1 ml. were used for each assay. The assays were heated at 100° and were started in such a way that all were completed at the same time. The assays were treated as described in the Materials and Methods. Percent degradation is the percent of the total recovered disintegrations/min. in peaks 1 and 2.

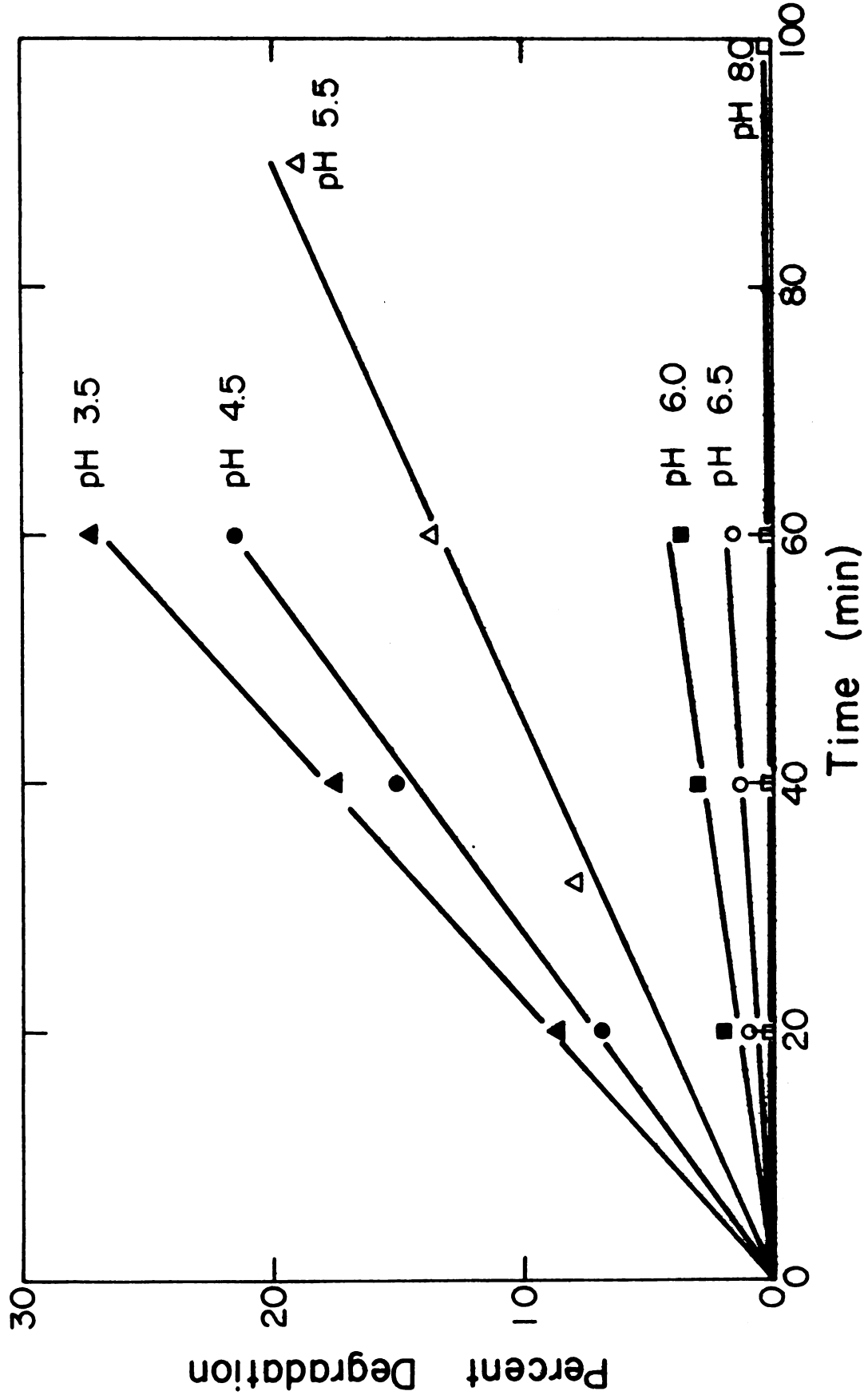
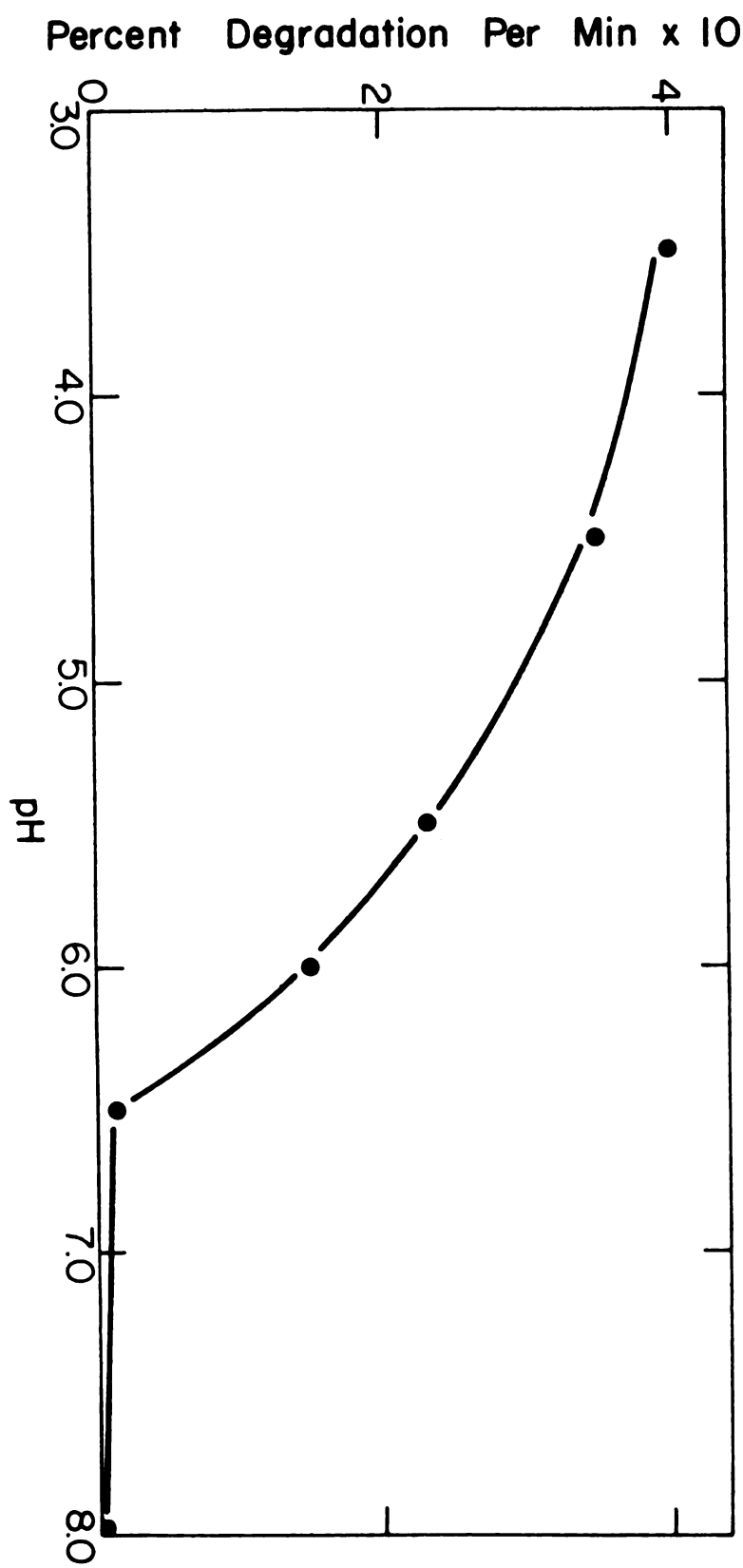

Percent Degradation
Time (min)
0
10
20
30
0
20
40
60
80
100
pH 3.5
pH 4.5
pH 5.5
pH 6.0
pH 6.5
pH 8.0

Figure 4. The rate of degradation of [^{14}C] apiogalacturonan 22SCS-IIa as a function of pH.

The rates of degradation were calculated from the data depicted in Figure 3.


Percent Degradation Per Min x 10
0
2
4
pH
3.0
4.0
5.0
6.0
7.0
8.0

the degradation as the pH of the solution decreased (Table 1). As the pH of the solution was decreased from 5.5 to 3.5, there was an increase in the amount of peak 1 in relation to peak 2 as well as an increase in the total percent degradation.

The rate and limit of the degradation were slightly inhibited in the presence of salt (Figure 5). The limit of the degradation obtained in 0.2 M-potassium phosphate buffer, pH 4.5, was 90% of that obtained in water.

The rate of the degradation was also a function of the concentration of the apiogalacturonan (Figures 6 and 7). The rate decreased as the concentration decreased from 10 mg./ml. to 1 mg./ml. The rate obtained at 1 mg./ml. was 67% of that obtained at 10 mg./ml. There was also an decrease in the ratio of peak 2 in relation to peak 1 as the concentration increased. After 90 min. at 100°, the ratio was 10.4, 8.2 and 6.5 at 1 mg./ml., 5 mg./ml. and 10 mg./ml., respectively. The degradation resulting with concentrations below 1 mg./ml. could not be measured accurately and concentrations above 10 mg./ml. were prohibitive because of the quantity of material needed for such assays.

Heating sodium [^{14}C] apiogalacturonate 22SCS-IIa in 0.5% ammonium oxalate, pH 6.2 (3 mg./ml.), for 6 hr. at 70° resulted in 3.75% degradation.

Table 1. The degradation products of [^{14}C] apiogalacturonan 22SCS-IIa as a function of pH.

Aliquots (0.1 ml.) of the sodium apiogalacturonate were heated for 180 min. at 100° at pH 3.5, 4.5, 5.5, 6.0, and 6.5 and at a concentration of 3 mg./ml. The individual assays were treated as described in the Materials and Methods. Percents are expressed as the percent of the total recovered disintegrations/min.

pH	Peak 1	Peak 2	$\frac{\text{Peak 2}}{\text{Peak 1}}$	Total degradation
	(%)	(%)		(%)
3.5	10.5	27.8	2.64	38.3
4.5	6.3	30.9	4.91	37.2
5.5	3.1	26.4	8.52	29.5
6.0	1.7	8.4	4.94	10.1
6.5	0.7	3.0	4.28	3.7

Figure 5. The effect of salt concentration on the degradation of [^{14}C] apiogalacturonan 22SCS-IIa.

The sodium salt of the polysaccharide was dissolved in water (●), 0.1 M- (O), and 0.2 M- (▲) potassium phosphate, all at pH 4.5 and at a concentration of 3 mg./ml. Aliquots of 0.1 ml. were used for each assay. The assays were heated at 100° and were started in such a way that all were completed at the same time. The assays were treated as described in the Materials and Methods. Percent degradation is the percent of the total recovered disintegrations/min. in peaks 1 and 2.

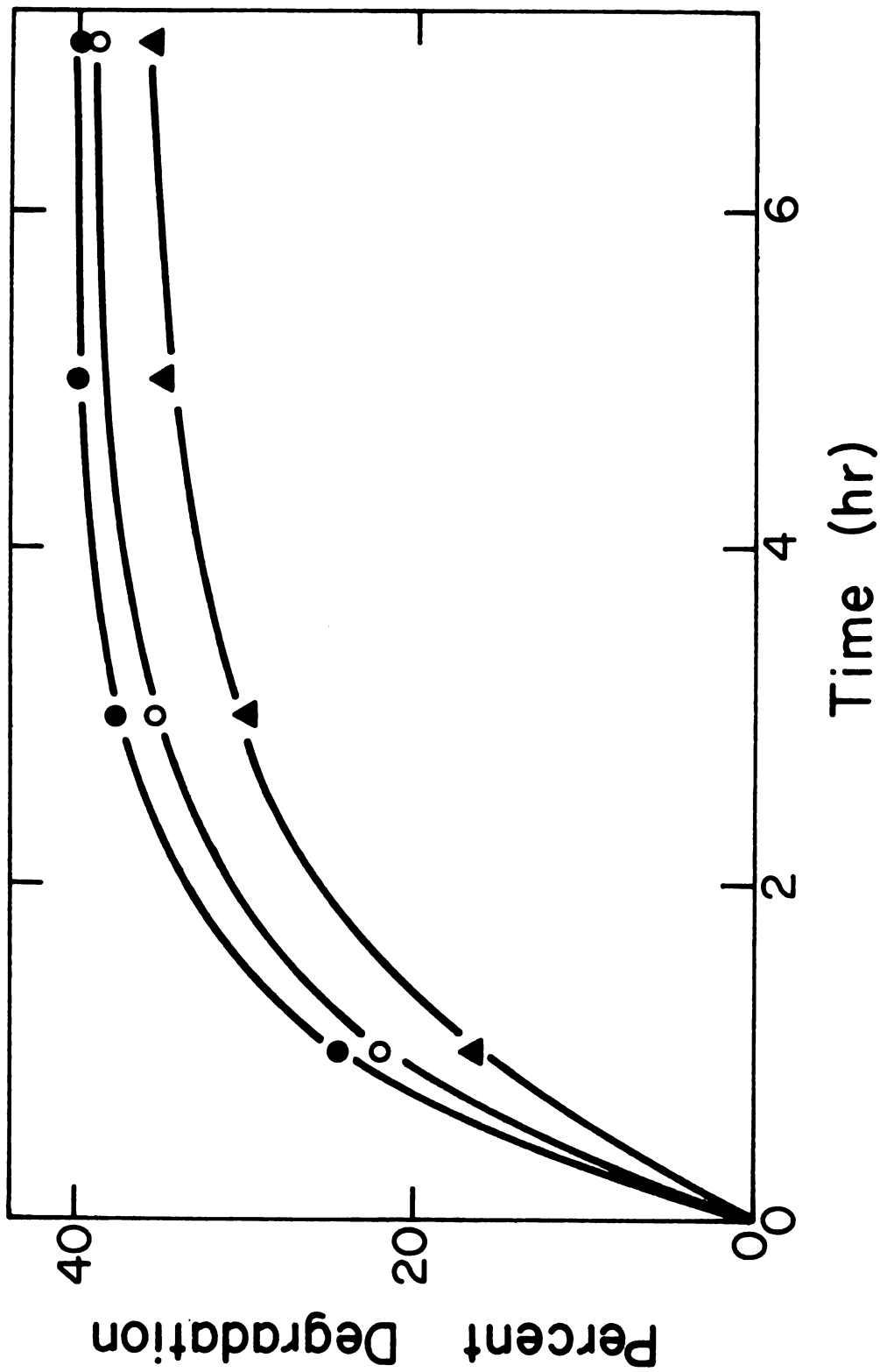

Percent Degradation
0
20
40
0
2
4
6
Time (hr)

Figure 6. The rate of degradation of [^{14}C] apiogalacturonan 22SCS-IIa as a function of concentration.

The sodium salt of the polysaccharide was dissolved in water at pH 4.5, at concentrations of 1.0 (●), 5.0 (△), and 10.0 (○) mg./ml. The assays were heated at 100° and were started in such a way that all were completed at the same time. The assays were treated as described in the Materials and Methods. Percent degradation is the percent of the total recovered disintegrations/min. in peaks 1 and 2.

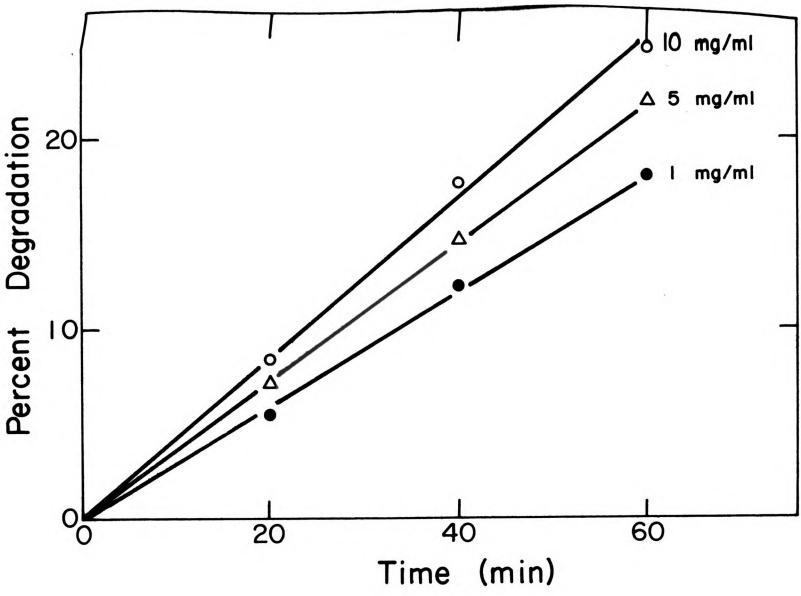
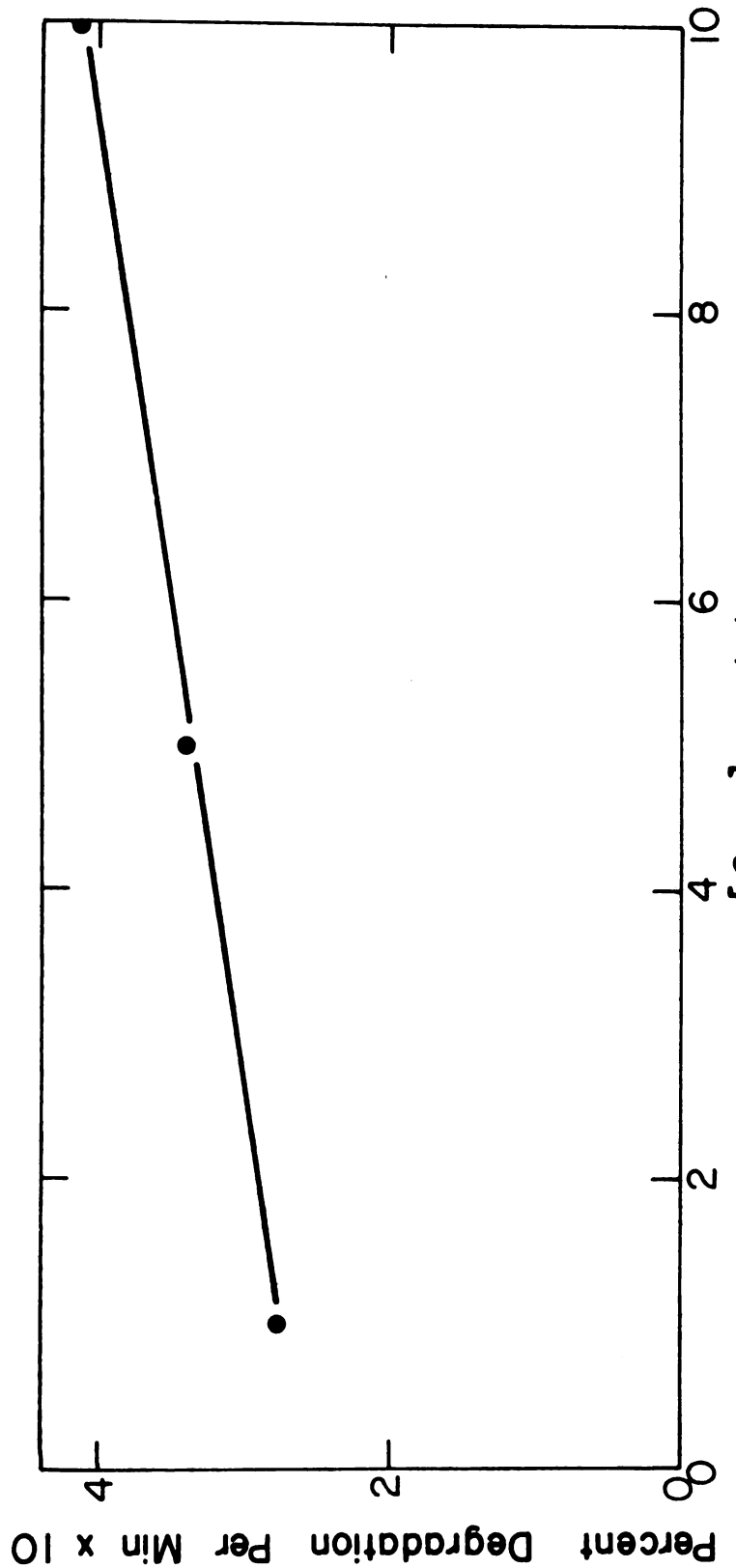

10 mg/ml
5 mg/ml
1 mg/ml
Percent Degradation
0
10
20
0
20
40
60
Time (min)

Figure 7. The rate of degradation of [^{14}C] apiogalacturonan 22SCS-IIa as a function of concentration.

The rates of degradation were calculated from the data depicted in Figure 6.


Percent Degradation Per Min x 10
0
2
4
2
4
6
8
10
[Conc] mg/ml

Identification of Radioactive Peak 1

Peak 1 was chromatographed in solvents A, B, C, and D. In all four solvents, it migrated as a single peak with the same R_F as D-apiose. When chromatograms were treated with aniline hydrogen phthalate, peak 1 developed as a yellow spot which fluoresced when viewed with ultraviolet light (max. 366 mμ). Both of these results are characteristic of D-apiose. Therefore it was concluded that peak 1 was D-apiose.

Identification of Radioactive Peak 2

Peak 2 chromatographed as a single peak in solvents A, B, C, and D. It had an $R_{D\text{-apiose}}$ of 0.53, 0.65, 0.75, and 0.95 in solvents A, B, C, and D, respectively. When chromatograms were treated with aniline hydrogen phthalate, peak 2 developed as a yellow spot which fluoresced when viewed with ultraviolet light (max. 366 mμ). On hydrolysis with 0.1 N-sulfuric acid, peak 2 was converted quantitatively to a compound which was chromatographically identical to D-apiose. The crystalline α-benzyl-α-phenylhydrazone derivative of this compound had a melting point of 137-138° and a $[\alpha]_{546}^{22}$ -86.3° (C2.7, pyridine). The same derivative of D-apiose had a melting point of 137-138° and $[\alpha]_{546}^{22}$ -90.1° (C4, pyridine). The mixed melting point of these two derivatives was 137-138°. Acid hydrolysis of sodium borohydride reduced peak 2 resulted in the release of equimolar quantities of D-apiose and D-apiitol. Similar hydrolysis

of the phenylosotriazole derivative of peak 2 resulted in the release of equimolar quantities of D-apiose and D-apiose phenylosotriazole. On the basis of these data, it was concluded that peak 2 was a disaccharide of D-apiose (apibiose).

Chemical Characterization of Apibiose

Both apibiose and apibiose phenylosotriazole were oxidized with sodium metaperiodate and the amount of formaldehyde released was determined. The results of these experiments may be seen in Table 2. The free disaccharide was oxidized with the release of 1 mole of formaldehyde per mole of disaccharide. Periodate oxidation of apibiose phenylosotriazole resulted in the release of 2 moles of formaldehyde per mole of derivative.

Methylation analysis of apibiose and apibiose phenylosotriazole resulted in the isolation of a single methylated sugar from the latter and two methylated sugars from the former. The fast migrating component from methylated apibiose and the methylated sugar component from methylated apibiose phenylosotriazole were indistinguishable from 2,3,3'-tri-O-methyl-D-apio-D-furanose as determined by paper and gas-liquid chromatography (Table 3). These methylated sugars also co-chromatographed with 2,3,3'-tri-O-methyl-D-apio-D-furanose as a single undistorted peak in the gas-liquid system.

Table 2. Periodate oxidation of apibiose and apibiose phenylosotriazole and determination of formaldehyde.

Periodate oxidations and determination of formaldehyde were performed as described in the Materials and Methods.

Compound	μmoles Compound	μmoles H_2CO	moles H_2CO / mole of compound
Apibiose			
Expt 1	62.41	69.56	1.11
Expt 2	38.30	39.73	1.04
Apibiose Phenylosotriazole			
Expt 1	13.83	28.20	2.04
Expt 2	11.17	22.97	2.06
Expt 3	10.90	22.13	2.03
Expt 4	17.70	35.92	2.02

Table 3. Chromatography of methylated D-apiose from apibiose, apibiose phenylosotriazole, and apiin.

The methylated sugars were prepared as described in the Materials and Methods.

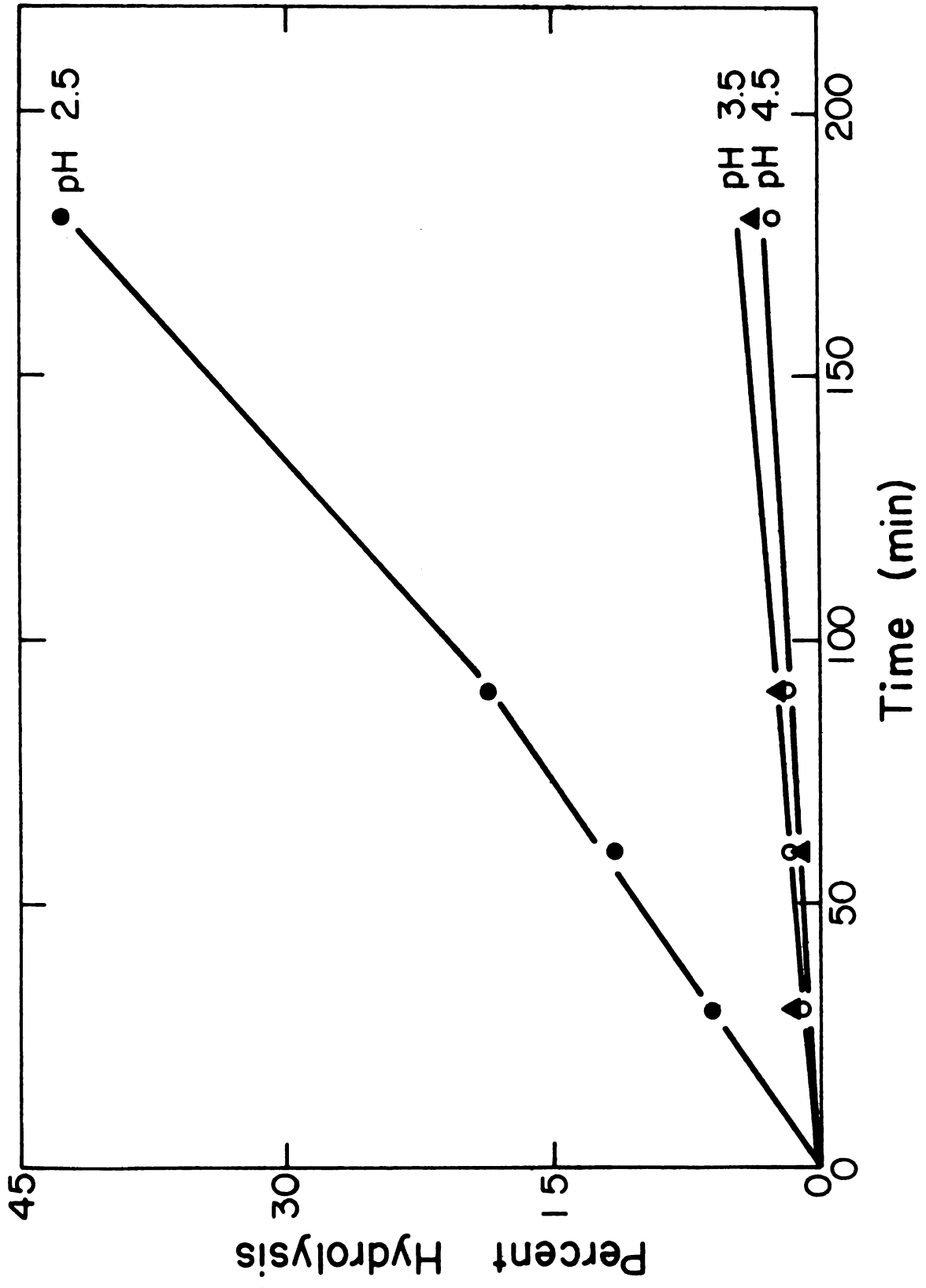
Solvent system	Apiin[a]	Apibiose phenylosotriazole	Apibiose	
			1	2
Paper Chromatography				
A	0.95	0.96	0.96	0.83
E	0.96	0.94	0.94	0.85
F	0.97	0.97	0.97	0.74
G	0.93	0.92	0.93	0.82
Gas-Liquid Chromatography	0.32	0.32	0.32	--

Note: Paper chromatographic mobilities are quoted relative to 2,3,4,6-tetra-O-methyl-D-glucose. Retention times are quoted relative to the same standard.

[a]2,3,3'-tri-O-methyl-D-apio-D-furanose.

Figure 8. Hydrolysis of [^{14}C] apiose in 0.05 M-potassium phosphate at pH 2.5 (●), 3.5 (▲), and 4.5 (○).

Aliquots (0.2 ml.) of a 1 mg./ml. solution were heated at 100°. The assays were started in such a way that all were completed at the same time. The assays were treated as described in the Materials and Methods. Percent hydrolysis is the percent of the total recovered disintegrations/min. in D-apiose.


Percent Hydrolysis
45
30
15
0
0
50
100
150
200
Time (min)
pH 2.5
pH 3.5
pH 4.5

Hydrolysis of Radioactive Apibiose

Radioactive apibiose was hydrolyzed with 0.05 M-potassium phosphate at pH 2.5, 3.5, and 4.5. As the results in Figure 8 indicate, after 180 min. at 100°, there was very little hydrolysis at pH 4.5 and 3.5 (2.4 and 3.7%, respectively) while at pH 2.5 there was 42% hydrolysis.

Physical Characterization of Apibiose

The infrared spectrum of apibiose was identical to that obtained for D-apiose except for an absorption band at 825 cm^{-1} in the apibiose spectrum (Figure 9). The infrared spectra of the phenylosotriazole derivatives of these two compounds (Figure 10) were more complex than the free sugars. The spectrum of apibiose phenylosotriazole revealed bands at 825 cm^{-1} and 1278 cm^{-1} which were absent from the D-apiose phenylosotriazole spectrum. The spectrum of the D-apiose phenylosotriazole revealed an intense band at 1232 cm^{-1} which was absent from the spectrum of apibiose phenylosotriazole. The spectra between 4000 cm^{-1} and 1400 cm^{-1} of all of these compounds was non-informative and contained no distinguishing bands.

The proton magnetic resonance spectrum of apibiose phenylosotriazole may be seen in Figure 11. There was no detectable resonance between 0.0-1.3 τ and 6.3-10.0 τ. The spectrum contained two distinct regions. Integration of the 1.5-2.3 τ region indicated that this region contained

Figure 9. Infrared spectrum of D-apiose and apibiose.

The amount of sample in the potassium bromide pellets was 1% (w/w). See Materials and Methods for experimental details.

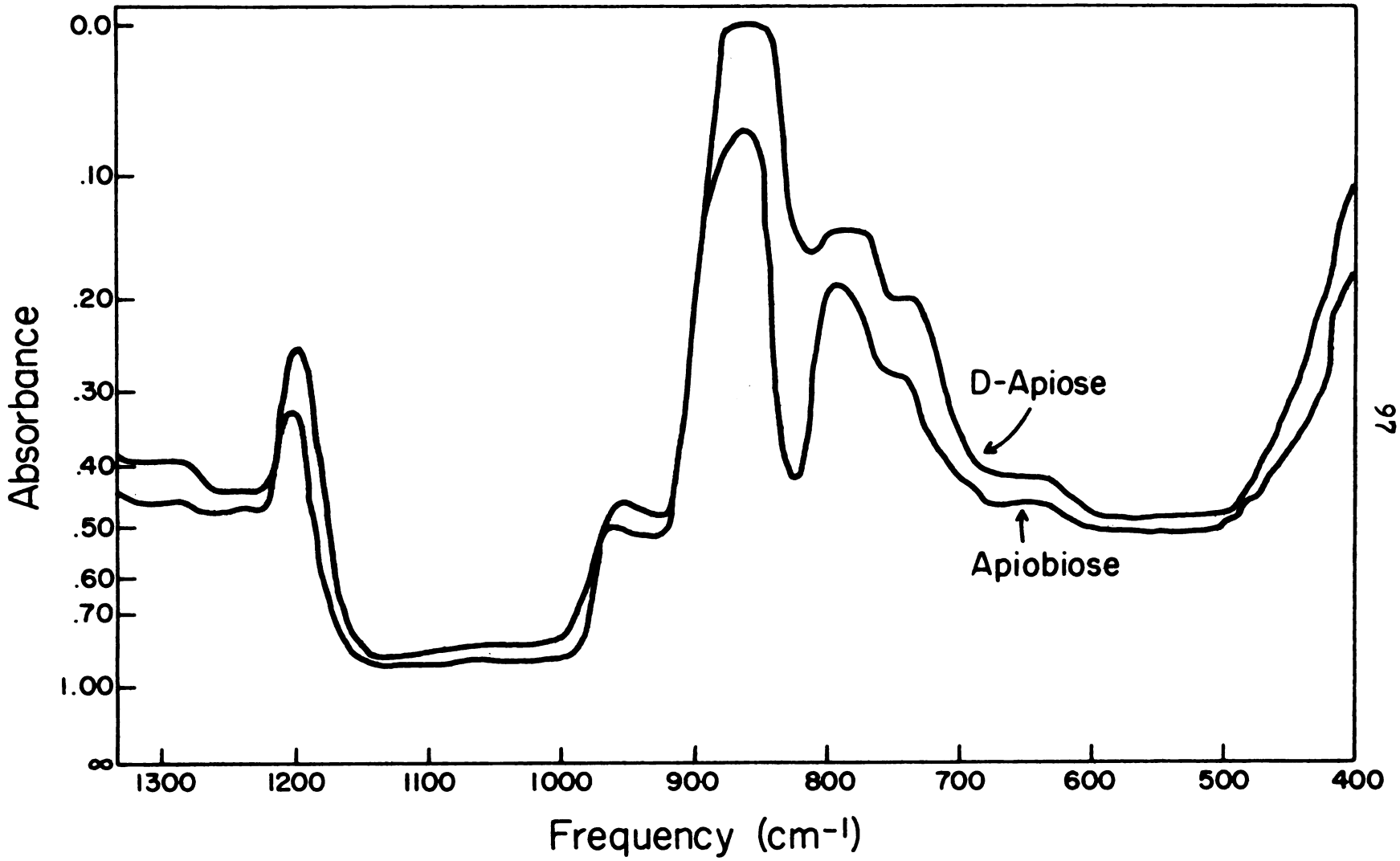

0.0
.10
.20
.30
.40
.50
.60
.70
1.00
∞
Absorbance
1300
1200
1100
1000
900
800
700
600
500
400
Frequency (cm-1)
D-Apiose
Apiobiose

Figure 9. Infrared spectrum of D-apiose and apibiose.

The amount of sample in the potassium bromide pellets was 1% (w/w). See Materials and Methods for experimental details.

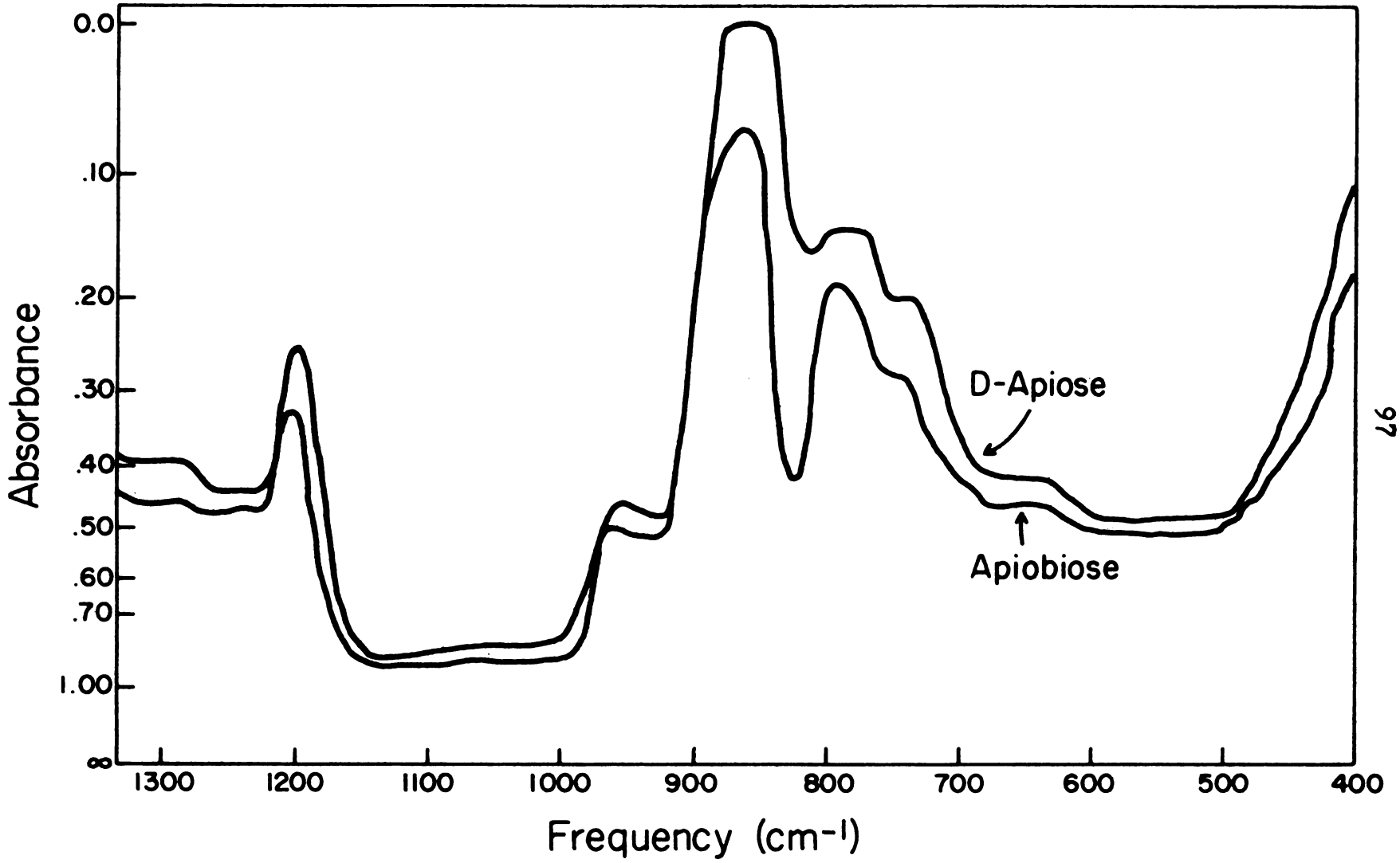

0.0
.10
.20
.30
.40
.50
.60
.70
1.00
∞
Absorbance
1300
1200
1100
1000
900
800
700
600
500
400
Frequency (cm-1)
D-Apiose
Apiobiose

Figure 10. Infrared spectrum of D-apiose phenylosotriazole (---) and apibiose phenylosotriazole (——).

The amount of sample in the potassium bromide pellets was 0.5% (w/w). See Materials and Methods for experimental detail.

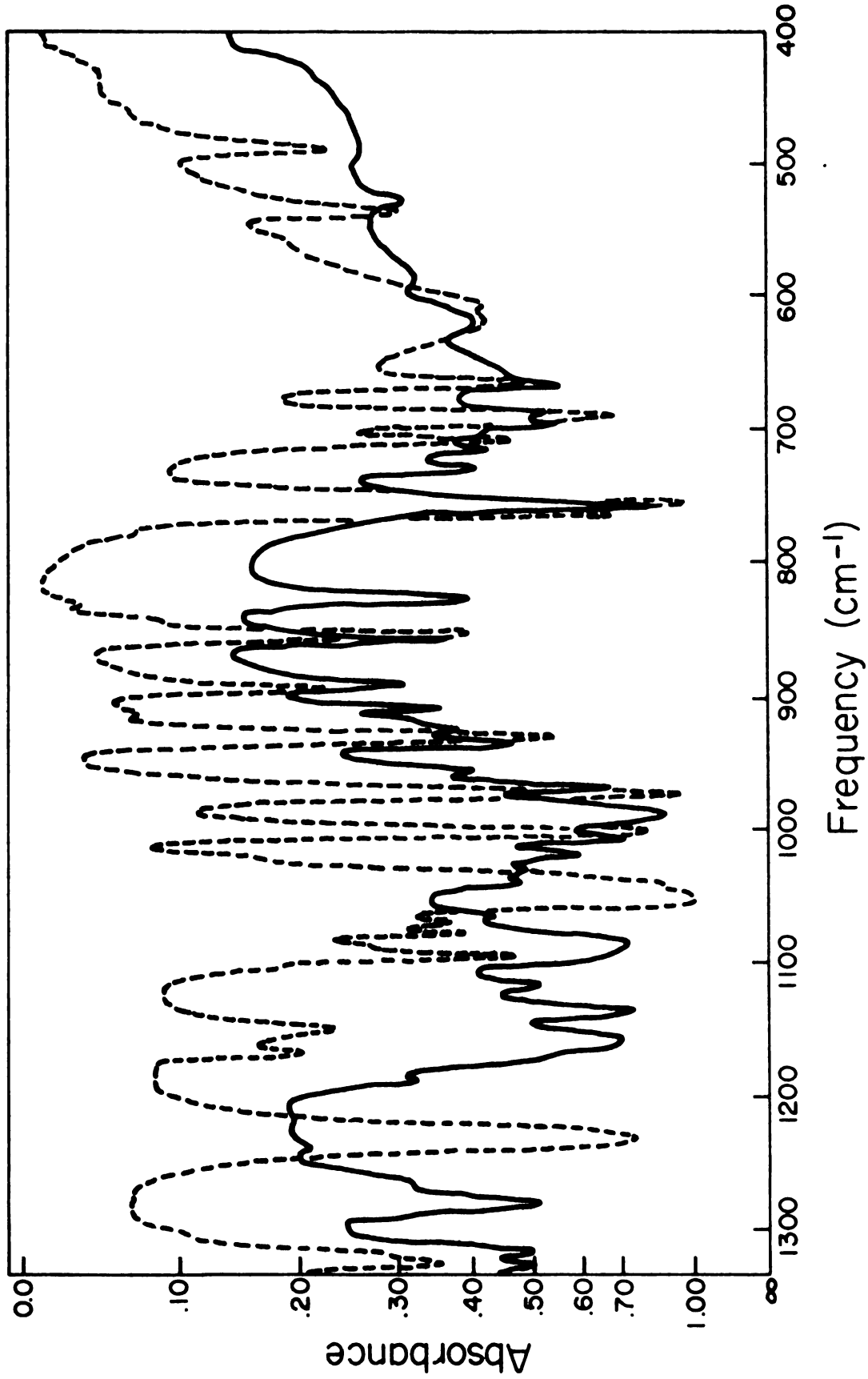
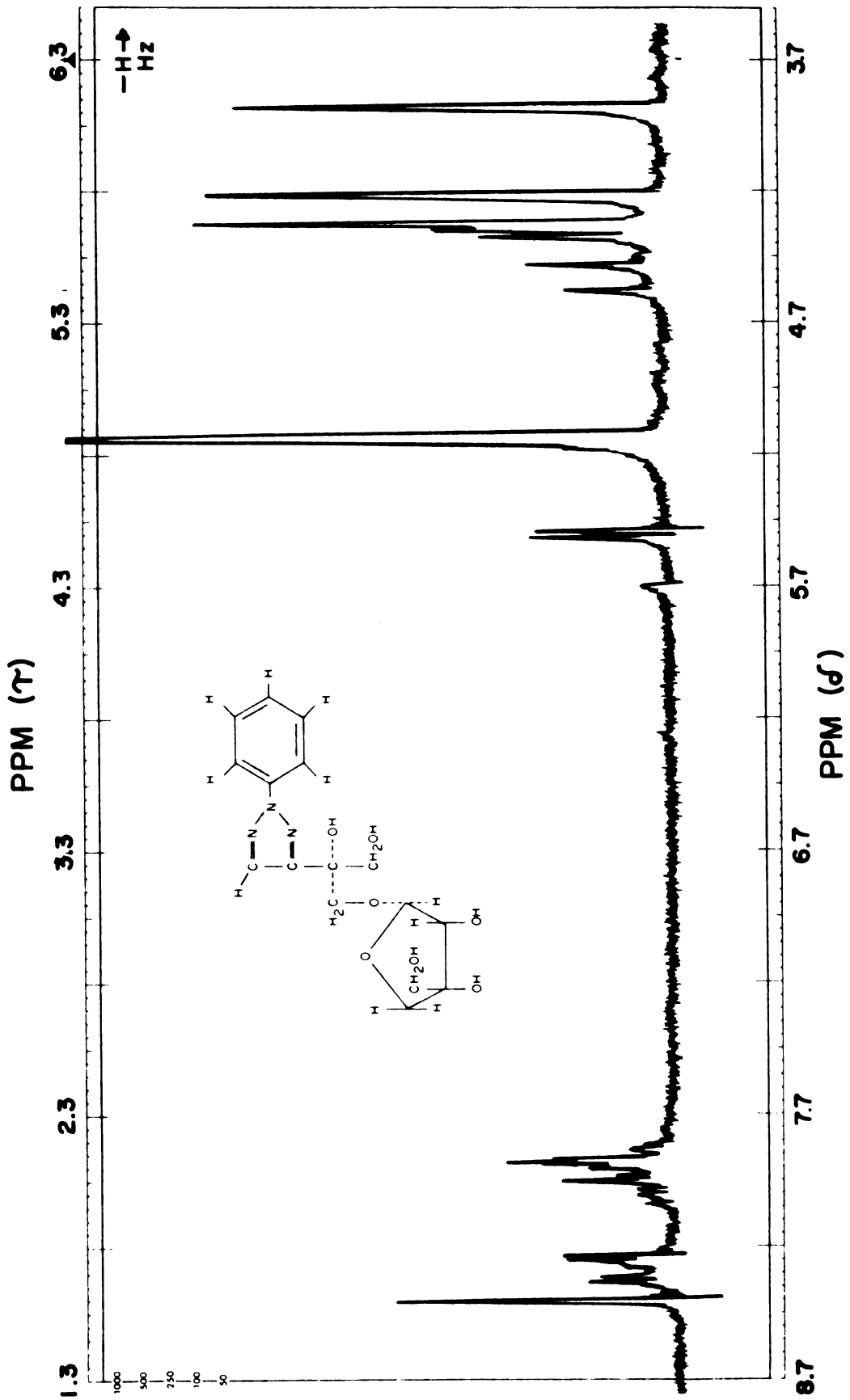

Absorbance
0.0
.10
.20
.30
.40
.50
.60
.70
1.00
Frequency (cm⁻¹)
400
500
600
700
800
900
1000
1100
1200
1300

Figure 11. Proton magnetic resonance spectrum of apibiose phenylosotriazole in deuterium oxide (10%, w/v).

The spectrum was obtained at 100 MHz.


PPM (τ)
PPM (δ)
1.3
2.3
3.3
4.3
5.3
6.3
8.7
7.7
6.7
5.7
4.7
3.7
—H→
Hz
1000
500
250
100
50
CH2OH
OH
OH
CH2OH

six protons. There was a one proton singlet at 1.59 τ, a two proton multiple at 1.63-1.78 τ, and a three proton multiple at 1.82-2.20 τ. The other region at 4.3-6.3 τ contained the remaining ten protons. This region contained a one proton doublet (J = 2.6 Hz) at 4.50 τ, a large HOD peak at 4.87 τ, a partially resolved area at 5.3-5.8 τ, and a two proton singlet at 6.11 τ. Expansion of the partially resolved area (5.3-5.8 τ) of the spectrum led to the increased resolution seen in Figure 12. This area of the spectrum contained a two proton singlet at 5.78 τ, a two proton singlet at 5.67 τ, a one proton doublet (J = 2.6 Hz) at 5.64 τ, and a two proton AB quartet (J = ~10.0 Hz) at 5.62 τ. The results are summarized in Table 4.

Mass spectral analysis of apibiose phenylosotriazole did not yield a molecular ion under the conditions employed for ionization. However, there was an intense peak at m/e 336 which corresponds to M-31. Under the same conditions, a molecular ion was obtained for D-apiose phenylosotriazole. The mass spectrum obtained with apibiose phenylosotriazole recovered from deuterium oxide was identical to that obtained with material which was never in deuterium oxide. This result indicated that there was no permanent exchange of C-H hydrogens during the recording of the proton magnetic resonance spectrum.

Figure 12. Proton magnetic resonance spectrum of apibiose phenylosotriazole between 6.35-5.35 τ.

The spectrum was obtained at 100 MHz.

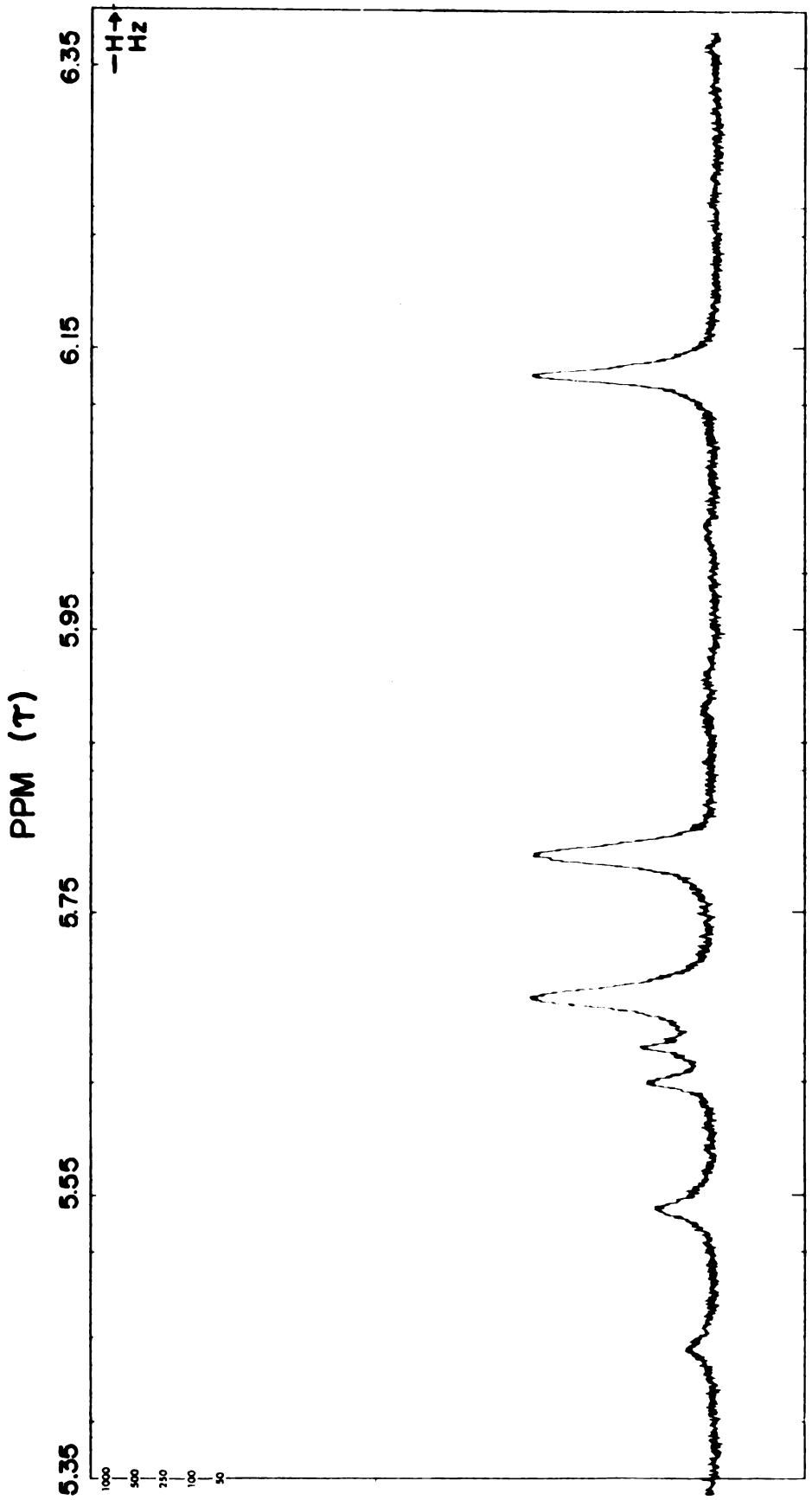
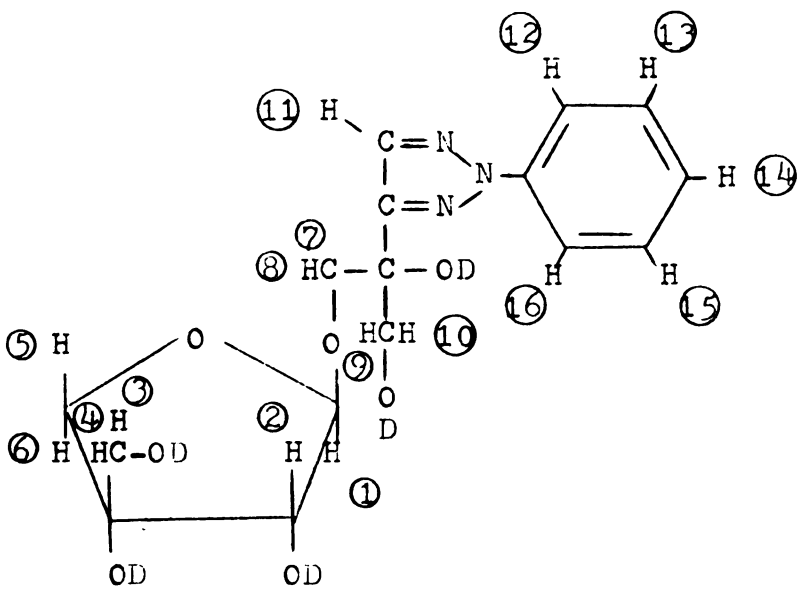

PPM (τ)
5.35
5.55
6.75
5.95
6.15
6.35
PPM (δ)
4.65
4.45
4.25
4.05
3.85
3.65
—H→ Hz
1000
500
250
100
50

Table 4. Summary of the proton magnetic resonance data obtained at 100 MHz for apibiose phenylosotriazole in deuterium oxide.

Peak assignments are numbered according to the diagram below.

Position (τ)	Number of protons	Type	Coupling constant (Hz)	Assign.
1.59	1	singlet	--	H-11
1.63-2.20	5	multiple	--	H-12,13 14,15,16
4.50	1	doublet	2.6	H-1
5.62	2	AB quartet	10.0	---[a]
5.64	1	doublet	2.6	H-2
5.67	2	singlet	--	---[a]
5.78	2	singlet	--	---[a]
6.11	2	singlet	--	---[a]

[a]These signals are from protons 3,4; 5,6; 7,8; or 9,10.



Characterization of Partially Degraded [^{14}C] Apiogalacturonans

When partially degraded [^{14}C] apiogalacturonans were chromatographed in solvent A, all of the radioactive material remained at the origin. Pectinase hydrolysis of partially degraded [^{14}C] apiogalacturonans resulted in a 75% conversion to D-galacturonic acid. Further characterization was not attempted.

DISCUSSION

The apiogalacturonan (22 3CS-IIa) used in most of this work is a representative member of the group of apiogalacturonans isolated from L. minor (Part II). All had a low content of esterified D-galacturonic acid residues. The low ester content and the pH profile depicted in Figure 4 for the thermal degradation reaction of this apiogalacturonan indicate that the degradation reaction reported here is a hydrolysis reaction rather than an elimination reaction such as that described for pectins by Albersheim (1959). The failure to detect any unsaturated degradation products and the ready conversion of the galacturonan residue to D-galacturonic acid by pectinase treatment also speak against an elimination reaction. However it does not appear to be a simple acid hydrolysis reaction. The relatively mild acid conditions needed for degradation suggest that the reaction may be an intramolecular hydrolysis involving the free

carboxyl groups of the D-galacturonic acid residues. The stability of apibiose to hydrolysis at pH 3.5 and 4.5, conditions which result in the rapid release of this compound from the apiogalacturonans, supports this suggestion. However, the rate of the degradation and the ratio of the degradation products were also a function of the apiogalacturonan concentration, results which indicate that there may be some intermolecular hydrolysis.

The disaccharide of D-apiose was the main degradation product isolated. Whether the D-apiose in the apiogalacturonans exists entirely as disaccharide sidechains which are then partially hydrolyzed to D-apiose during and/or after hydrolysis from the apiogalacturonans, cannot be determined from the data. The periodate oxidation data reported in Table 7, Part II, also indicate that the D-apiose is almost entirely in the disaccharide form. The limit of the percent degradation for all of the apiogalacturonan fractions approached the percent D-apiose of these fractions, indicating that whatever the mechanism, the D-apiose appears to be attached to the galacturonan backbone in a similar manner in all of the fractions.

All of the above described data lead to the conclusion that there is an unusual structural feature of these apiogalacturonans which causes the apibiose:galacturonan glycosidic linkage to be extremely susceptible to hydrolysis. The apibiose can only be linked to the D-galacturonic acid

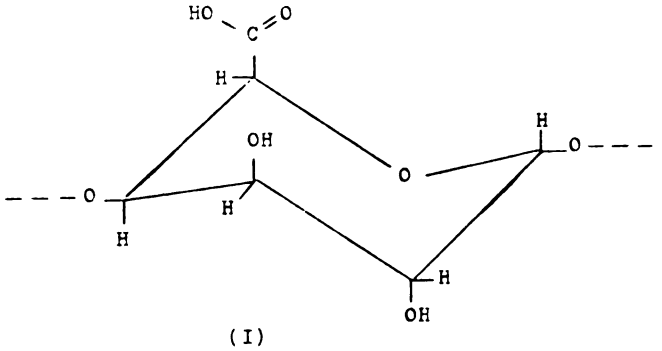
residues at position 2 and/or 3 since the galacturonan is linked α-(1→4). If the apibiose was attached to the hydroxyl group at C-3 and the D-galacturonic acid residues were in the conformation indicated (I), it would then be in close proximity to the carboxyl group. However, this structure and conformation may not be required for hydrolysis since there may be deformation of the apiogalacturonan due to the heating which could result in conditions favorable to hydrolysis when the apibiose is attached to C-2 of the D-galacturonic acid residues. It would be of interest to determine if the galacturonans isolated by Bouveng (1965), Aspinall and Baillie (1963) and Aspinall and Fanshawe (1961), which also contain a large percentage of neutral sugars as side-chains, also undergo a degradation of the type reported here.

The thermal degradation of apiogalacturonan 22SCS-IIa in 0.5% (w/v) ammonium oxalate, pH 6.2, was very slight, being 3.75% after 6 hr. at 70°. In Part II, the possibility of the partial degradation of the 70° ammonium oxalate fractions due to the elevated temperatures was noted. This result indicates that any degradation of the type described in this report was probably minor.

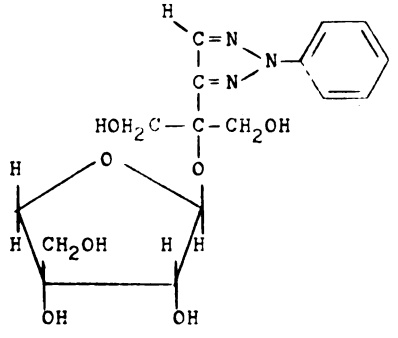
The failure to detect apibiose or D-apiose when the residue fraction was heated does not imply that the remaining 76% (Table 1, Part II) of the D-apiose of the cell wall is in a different form. First, the degradation may not

occur in the solid state where the carboxyl groups may be unavailable due to complexing with cations. Secondly, if the carboxyl groups of uronans have an essential role in the degradation of the apiogalacturonans and the D-apiose remaining in the cell wall is a component of polysaccharides which contain only neutral sugars then these polysaccharides would not undergo a degradation such as described here. Therefore nothing may be stated at this time as to the state of the D-apiose remaining in the residue fraction.

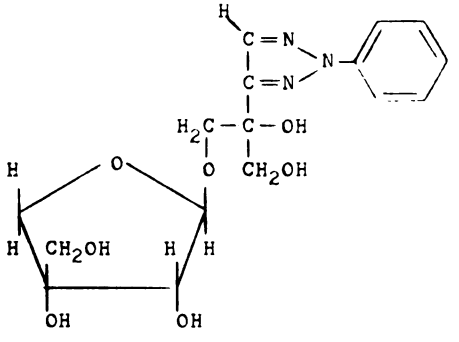
Apibiose was oxidized by sodium metaperiodate with the release of 1 mole of formaldehyde per mole of disaccharide. This result and the periodate results obtained earlier with intact apiogalacturonans (Table 5, Part 2), indicate that the glycosidic linkage between the two D-apiose molecules is either 1→3 or 1→3'. It is of interest to note that this result with apibiose also indicates that the intermediate formyl ester (IV) was essentially stable under the conditions of the oxidation. The data obtained from the periodate oxidation of apibiose phenylosotriazole permitted a choice to be made between the two linkage alternatives. Periodate oxidation of (II) should result in the release of one equivalent of formaldehyde while the oxidation of (III) should result in the release of two equivalents of formaldehyde. The data in Table 2 show that two equivalents of formaldehyde were released. Therefore the glycosidic linkage between the two D-apiose molecules is 1→3' and



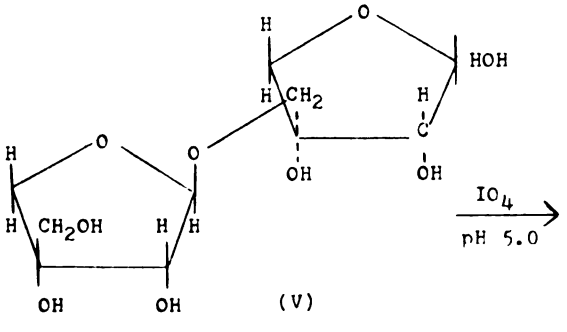
(I)



(II)



(III)



(V)

(IV)

structure (III) is the correct one for apibiose phenylosotriazole.

The methylation data indicate that the non-reducing terminal D-apiose of apibiose has the D-apio-D-furanose configuration. This is the same configuration as that obtained by Halyalker et al. (1965) for the D-apiose in apiin, the only other naturally occurring compound for which the configuration at C-3 of D-apiose has been determined. The stereochemistry of the reducing terminal D-apiose at C-3 was not established. However, since apparently the natural isomer of D-apiose is the D,D isomer, the reducing terminal D-apiose may also have the D-apio-D-furanose configuration. The slower migrating component from the hydrolysates of methylated apibiose is from the reducing end of the disaccharide. The methylated compound is 2,3-di-O-methyl-D-apiose.

Molecular rotations have been used to indicate the stereochemistry of anomeric linkages. Halyalker et al. (1965) extrapolated the theory of Klyne (1950) to the case of apiin and concluded that the linkage of the D-apiose to the 7-O-D-glucosyl-apigenin had the β configuration. Klyne's theory states that the contribution of a carbohydrate moiety to the total molecular rotation of a compound is approximately equal to the molecular rotation of the methyl glycoside of the carbohydrate moiety. That is

$$\Delta \text{ rotation} = [M] \text{ R-carbohydrate} - [M] \text{ R}$$

$$= [M] \text{ methyl glycoside of carbohydrate}$$

For sugars of the D-series, the α-methyl glycosides have a more positive rotation than the β-methyl glycosides. In fact, the α-methyl glycosides of D-sugars usually have a positive rotation or a rotation close to zero while β-methyl glycosides have rotations of -100° to -300°. The molecular rotations of identified methyl glycosides of D-apiose have not been determined. Williams and Jones (1964) isolated a chromatographically pure methyl glycoside of D-apiose which had a $[\alpha]_D^{24}$-101° ($[M]_D^{24}$-165.6°). However they did not characterize the configuration at C-1 or C-3. Those isopropylidene derivatives of D-apiose which have the α configuration all have a positive rotation (Carey, Ball and Long, 1966, and Ball, Carey, Klundt and Long, 1969).

The theory of Klyne (1950) is not directly applicable to the determination of the anomeric configuration of D-apibiose phenylosotriazole since the attachment of the second D-apiose molecule to D-apiose phenylosotriazole creates a new asymmetric center at C-3 of the D-apiose phenylosotriazole component. The observed molecular rotation for apibiose phenylosotriazole, -294°, is due to the second D-apiose molecule plus the component due to the asymmetric center at C-3 of the D-apiose phenylosotriazole. Since its rotation is zero, D-apiose phenylosotriazole cannot be used to assess the rotation contribution of the assymmetric center at C-3. D-Erythrose phenylosotriazole

could be used, however this compound is not known.

The case of the free apibiose is different from that of apibiose phenylosotriazole. Since there was no detectable mutarotation, the configuration at the reducing end of the molecule must have attained equilibrium before the compound was concentrated to a syrup. The molecular rotation of equilibrated D-apiose is only slightly positive (less than 10°) (Schmidt, 1930). Therefore, keeping in mind that the reducing terminal D-apiose molecule can only equilibrate between three forms whereas free D-apiose can equilibrate between five forms, one may still conclude that the major component of the observed rotation of the disaccharide, $[M]_{578}^{22}$ -194.9°, is probably due to the non-reducing D-apiose molecule. This means that this molecule is contributing a negative rotation.

In view of the positive rotations for the isopropylidene derivatives of D-apiose, which have the α configuration and the fact that methyl α-D-erythrofuranoside and β-D-erythrofuranoside have molecular rotations of approximately $+199^{\circ}$ and -217°, respectively (Halyalkar, Jones and Perry, 1965), the negative rotations obtained for apibiose and apibiose phenylosotriazole indicate that the anomeric linkage has the β configuration since it is doubtful that the α-methyl glycoside of D-apio-D-furanose would have such a large negative rotation.

Assignment of protons to all of the signals in the proton magnetic resonance spectrum of apibiose phenylosotriazole cannot be made since the spectra of a sufficient number of model compounds have not been determined. The

assignments which have been made were based on literature precedents for proton magnetic resonance spectra of organic compounds in general (Silverstein and Bassler, 1964, and Morrison and Boyd, 1966, and references therein) and of carbohydrates specifically (Hall, 1964, and references therein).

From the proton magnetic resonance spectrum the apparent coupling constant between the H-1 and H-2 protons of the non-reducing D-apiose molecule was calculated to be 2.6 Hz. Assuming the modified constants of Abraham *et al.* (in Hall, 1964) for the Karplus equation (Karplus, 1959) are valid for this system, values of $56\pm5^{\circ}$ ($0^{\circ}\leq\emptyset\leq90^{\circ}$) and $121\pm5^{\circ}$ ($90^{\circ}\leq\emptyset\leq180^{\circ}$) were calculated for the dihedral angle between H-1 and H-2 (see Appendix for calculations). For a planar furanose ring the projected valency angles for *cis* hydrogens is 0° and for *trans* hydrogens is 120°. The only literature values for D-apiose containing compounds are those of Carey *et al.* (1966) and Ball *et al.* (1968). Working with the mono- and di-O-isopropylidene derivatives of D-apio-D-furanose and D-apio-L-furanose, having the α configuration at the anomeric carbon atom, these workers obtained values of 3.5-3.7 Hz for the apparent coupling constants between H-1 and H-2. They calculated that the dihedral angle between H-1 and H-2 is $40\text{-}50^{\circ}$. Therefore the formation of the fused ring system in these isopropylidene derivatives resulted in the D-apiose adopting the

"twist" conformation in which C-2 was below the plane formed by C-1, C-4 and the ring oxygen, and C-3 was above this plane (Hall, 1964).

For apibiose phenylosotriazole, the value of 56 ± 5^{o} for the dihedral angle indicates that the linkage in apibiose has the α configuration and the non-reducing terminal furanose ring also has the "twist" conformation. On the other hand, the value of 121 ± 5^{o} for the dihedral angle indicates that the linkage has the β configuration and that this part of the furanose ring is planar. The entire ring is probably not planar however. Jardetzky (1960) has shown that the ribofuranose ring in nucleosides is not planar. The interaction between the two cis-eclipsed hydroxyl groups at C-2 and C-3 are such that the furanose ring adopts a conformation to minimize this interaction. Results with carbohydrates in the furanose ring indicate they adopt the conformation that minimizes non-bonded interactions between adjacent substituents (Hall, 1964). Therefore, the configuration of the linkage between the two D-apiose molecules of apibiose may be β and the non-reducing terminal D-apiose molecule has adopted one of the other possible conformations for the furanose ring (Hall, 1964).

Therefore, the available data permit a tentative assignment of the β configuration for the anomeric linkage between the two D-apiose molecules of apibiose and the structure of the disaccharide is 3'-O-D-apio-D-furanosyl-D-

apiose (V). When attached as sidechains to the α-(1→4)-galacturonan, the disaccharide is (2 and/or 3)-O-[O-β-D-apio-D-furanosyl-(1→3')-(α or β)-D-apio-(D or L)-furanosyl]-galacturonan. Additional data, such as the molecular rotations and proton magnetic resonance spectra of the four methyl glycosides of D-apiose, is needed before this assignment can be substantiated.

It had been postulated by Beck (1967) that the D-apiose in apiogalacturonans, isolated from L. minor, was present as monomeric sidechains. However, his conclusion was based on hydrolysis data alone. It is possible that re-examination of his polysaccharide preparations by the periodate oxidation and the mild hydrolysis procedures reported here, may reveal the presence of disaccharide units of D-apiose.

There are a number of structural aspects of these apiogalacturonans which remain to be determined. These include the configuration at C-3 of the reducing terminal D-apiose of apibiose, the position and stereochemistry of the glycosidic linkage between apibiose and the galacturonan, determination of a repetitive sequences, if any, in the apiogalacturonans and molecular weight. These problems are now under study in preparation for investigating the biosynthesis of these polysaccharides.

APPENDIX

Calculation of Dihedral Angles With the Karplus Equation

From Karplus (1959):

$$J = J_o \cos^2 \phi + K$$

J = observed coupling constant

K = -0.28 Hz

ϕ = dihedral angle

J_o = constant dependent on the quadrant of ϕ

From Abraham *et al.* (in Hall, 1964):

(A) $J_o = 9.3$ for $0^\circ \leq \phi \leq 90^\circ$

(B) $J_o = 10.4$ for $90^\circ \leq \phi \leq 180^\circ$

(A) $J = 2.6$ Hz $\quad J_o = 9.3 \quad 0^\circ \leq \phi \leq 90^\circ$

$$2.6 = 9.3 \cos^2 \phi - 0.28$$

$$\cos^2 \phi = 0.31$$

$$\cos \phi = 0.557$$

$$\phi = 56^\circ$$

(B) $J = 2.6$ Hz $\quad J_o = 10.4 \quad 90^\circ \leq \phi \leq 180^\circ$

$$2.6 = 10.4 \cos^2 \phi - 0.28$$

$$\cos^2 \phi = 0.277$$

$$\cos \phi = -0.526$$

$$\phi = 121^\circ$$

The observed coupling constant is a time averaged constant since the molecule is in constant motion. Therefore the values calculated from the Karplus equation are only a time average approximation of the dihedral angle (Hall, 1964).

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