A Novel Reaction Involved in the Degradation of Apiogalacturonans from *Lemna minor* and the Isolation of Apibiose As a Product*

David A. Hart† and Paul K. Kindel‡

ABSTRACT: Apiogalacturonans isolated from the cell wall of *Lemna minor* were found to be degraded under unusually mild acid conditions. At pH 4.5 and 100°, this degradation reaction is virtually complete in 3 hr and is more than half-complete in 1 hr. The rate of the reaction at 80° is approximately one-third that at 100°. The rate of the reaction declined steadily from pH 3.5 to almost zero at pH 6.5. The major products of the reaction are a new disaccharide containing only D-apiose and a galacturonan. We give the common name apibiose to this new disaccharide. A small amount of D-apiose was also obtained. All cell wall fractions isolated from *L. minor* and known to contain apiogalacturonans also gave these same products when subjected to the above degradation conditions. The amount of apibiose and D-apiose released from an aprogalacturonan or from a cell wall fraction containing apiogalacturonans was approximately equivalent to its D-apiose content. This indicated that all the D-apiose in the polysaccharides was attached in a similar manner.

The postulation is made that the degradation reaction is a hydrogen ion catalyzed hydrolysis in which there is transannular participation of the free carboxyl group of the D-galacturonic acid residues in the cleavage of the glycosidic bond between the apibiose side chains and the galacturonan. Apibiose was characterized as the free sugar, as the corresponding sugar alcohol, named apibiitol, and as its crystalline phenylosotriazole derivative. These studies showed that apibiose was a disaccharide of D-apiose and that the position of the linkage between the two D-apiose molecules was 1→3′. Proton magnetic resonance spectra and molecular rotational data suggested that the glycosidic linkage had the β configuration. Apibiose therefore is *O*-β(?)-D-apio-(D or L)-furanosyl-(1→3′)-3-*C*-(hydroxymethyl)-*aldehydo*-D-*glycero*-tetrose.

The isolation and partial characterization of a series of apiogalacturonans from the cell wall of *Lemna minor* (duckweed) has been previously reported (Hart and Kindel, 1970). The results showed that the D-apiose (3-*C*-hydroxymethyl-*aldehydo*-D-*glycero*-tetrose) in these polysaccharides is glycosidically linked as side chains on galacturonans. Contrary to the conclusion of Beck (1967), the results indicated that the side chains are disaccharide units of D-apiose rather than monosaccharide units.

Further investigations on the structure of these apiogalacturonans have led to the isolation of a disaccharide of D-apiose. This disaccharide is released from the apiogalacturonans by unusually mild acid conditions. The present report is concerned with the isolation and structural characterization of this disaccharide and several parameters of the reaction involved in its release from the apiogalacturonans. We give the common name apibiose to this new disaccharide.

Materials and Methods

Materials. D-Apiose was obtained as described previously (Hart and Kindel, 1970). D-Apiitol was prepared from D-apiose (Neal and Kindel, 1970). D-Apiose phenylosotriazole was prepared by the method of Kindel (1970). [^{14}C]$BaCO_3$ was obtained from New England Nuclear Corp. and was converted into [^{14}C]$NaHCO_3$ (5 μCi/μmole) before use. Fungal pectinase was purchased from Sigma Chemical Co. (lot 125B-0350). The enzyme was purified 30-fold over the crude preparation by the manufacturer who stated it still contained several other enzymes which were not specified.

L. minor was grown on modified medium V of Norris *et al.* (1955) as described elsewhere (P. K. Kindel, manuscript in preparation). Large quantities were obtained from the Battle Creek River at Bellvue, Mich. The plants obtained from the river were washed extensively with water and either used immediately or stored at −20°.

Radioactive *L. minor* was obtained by exposing a starting quantity of 10 fronds in a closed container to increasing amounts of [^{14}C]CO_2 over a 30-day period. The plants were grown on the above medium at 22–24° under continuous incandescent light of approximately 100 ft-candles. The number of fronds doubled every 3 days. The [^{14}C]CO_2 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 container was kept closed for 24 hr after each administration of [^{14}C]CO_2 and was then opened to the atmosphere for the next 48 hr. A total of 415 μCi of [^{14}C]CO_2 was administered. Before use, the radioactive plants

* From the Department of Biochemistry, Michigan State University, East Lansing, Michigan 48823. *Received December 10, 1969.* This investigation was supported by Research Grant AM-08608 from the National Institutes of Health and by the Michigan Agricultural Experiment Station. This paper is Michigan Agricultural Experiment Station Journal Article 4934.

† National Aeronautics and Space Administration Fellow, 1964–1967. This paper is derived from a thesis submitted by D. A. H. to Michigan State University in partial fulfillment of the requirements for the degree Doctor of Philosophy. Present address: Department of Microbiology, University of Illinois College of Medicine, Chicago, Ill. 60680.

‡ To whom inquiries should be directed.

were diluted fivefold with nonradioactive *L. minor* grown under similar conditions.

Nonradioactive and radioactive cell wall fractions and sodium apiogalacturonans were isolated from nonradioactive and radioactive *L. minor*. The isolation procedure has been described previously (Hart and Kindel, 1970). The specific activities of the radioactive cell wall fractions and polysaccharides ranged from 14,000 to 18,000 dpm per mg. The *L. minor* cell wall fractions referred to throughout this paper are those listed in Table 1 of Hart and Kindel (1970). Because of the relative abundance, the high D-apiose content (38%), and the apparent homogeneity of the 22° sodium chloride soluble apiogalacturonan IIa (Table 2, Hart and Kindel, 1970), this polysaccharide prepared radioactive (referred to as sodium [^{14}C]apiogalacturonan IIa throughout this paper) was used for characterization of the degradation reaction. When rechromatographed on a DEAE-Sephadex column (Hart and Kindel, 1970), sodium [^{14}C]apiogalacturonan IIa eluted in the same position and as a single peak. The fractions were assayed for both uronic acid and D-apiose and both profiles were symmetrical and coincident. On this basis the polysaccharide was considered to be homogeneous. Homogeneous as used here means that the individual molecules of the polysaccharide have chemically identical repeating units but are not necessarily the same molecular weight.

General Methods. The procedure of Schmidt (1930) was used in the preparation of crystalline D-apiose α-benzyl-α-phenylhydrazone. Solutions were concentrated under reduced pressure by rotatory evaporation at bath temperatures less than 35°. Melting points are uncorrected. Optical rotations were determined with a Zeiss photoelectric precision polarimeter 0.005° (Carl Zeiss, Oberkochen, Germany) at 22° and at 578 and 546 nm. Molecular rotations were calculated from specific rotations by the following formula: $[M] = [\alpha] \times$ mol wt/100. Proton magnetic resonance spectra were obtained at 100 MHz with a Varian HA-100 spectrometer (Varian Associates). For spectral analysis samples were in D_2O at a concentration of 10% (w/v). Exchangeable hydrogens were removed by concentrating the samples several times from D_2O. The spectra were obtained at ambient temperatures with tetramethylsilane as the external standard ($\tau = 10$). Radioactivity was detected on chromatograms with a Packard radiochromatogram scanner, Model 7201 (Packard Instrument Co.). 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-*t*-butylbenzoxazolyl)]thiophene in reagent grade toluene (4 g/l.). The counting efficiencies with solutions A and B were 79 and 60%, respectively.

Paper Chromatography. Descending paper chromatography was used and was carried out with Whatman No. 3MM paper. The paper was prewashed with 0.1 M citric acid followed by distilled water unless otherwise stated. The following solvents were employed: (A) ethyl acetate–H_2O–acetic acid–formic acid (18:4:3:1, v/v), (B) 2-propanol–H_2O (9:1, v/v), (C) 1-propanol–ethyl acetate–H_2O (7:1:2, v/v), (D) pyridine–ethyl acetate–acetic acid–H_2O (5:5:1:3, v/v). Sugars were detected on chromatograms with aniline hydrogen phthalate (Partridge, 1949) or by the $AgNO_3$ dip method (Trevelyan *et al.*, 1950).

Degradation Experiments with [^{14}C]Apiogalacturonans. [^{14}C]-Apiogalacturonans were degraded in screw-top test tubes (13 mm o.d. × 100 mm) as described in the individual experiments reported. For each set of experiments the assays were started in such a way that all finished at the same time. The solutions were then spotted directly on unwashed paper. The papers were developed in solvent A, scanned, and the radioactive areas were cut out and counted directly in scintillation solution B. Only the three peaks shown in Figure 1a were ever obtained on the scans. Greater than 90% of the starting radioactivity could be accounted for in these three areas.

Isolation of Radioactive and Nonradioactive Apibiose. Radioactive or nonradioactive cell walls were isolated and then treated with 0.5% (w/v) ammonium oxalate, pH 6.2, at 22°. The material solubilized was precipitated once with $CaCl_2$, redissolved in 0.5% ammonium oxalate, and recovered by lyophilization. These procedures are described in detail in Hart and Kindel (1970). The material obtained following lyophilization was dissolved in water to give a 1% (w/v) solution having a pH of 4.3–4.5. The solution was heated for 3 hr at 100°, cooled, and concentrated to approximately a 3% (w/v) solution. Acetone (3 volumes) was poured slowly into the stirred solution. The resulting suspension was placed at 4° for 4 hr and then centrifuged at 15,000*g* for 20 min. After decantation of the supernatant solution, the precipitate was resuspended in 1 volume of acetone and the suspension was centrifuged. The combined supernatant solutions were analyzed for apibiose by paper chromatography. The precipitate was redissolved in water as described above and the above treatment repeated until only a small amount of apibiose was present in the supernatant solutions. For the radioactive apiogalacturonans, a single heat treatment was sufficient. In the larger scale nonradioactive isolations, three to six heat treatments were usually carried out. More highly purified cell wall fractions have been used for the isolation of apibiose with similar results. In one experiment sodium [^{14}C]apiogalacturonan IIa was used.

Radioactive apibiose was purified by preparative paper chromatography and then eluted with water. The disintegrations per minute per milliliter of concentrated eluate were determined with scintillation solution A. The [^{14}C]apibiose was assumed to be uniformly labeled.

Nonradioactive apibiose was purified by partition column chromatography on unwashed, standard grade, Whatman powdered cellulose. A modification of the procedure described by Whistler and BeMiller (1962) was used. Cellulose was suspended in acetone and the fines were removed. The column was poured with acetone and then washed and developed with water-saturated 1-butanol. Development was at a rate of 1.0 ml/min and 10-ml fractions were collected. When 1.5–2.0 g of impure apibiose was applied to a column of cellulose 5.0 cm in diameter and 40 cm high, there was complete separation of apibiose from D-apiose. Fractions were analyzed by spotting aliquots on filter paper and then visualizing the sugars. Apibiose was concentrated several times to a syrup from water and then filtered through a Seitz filter. After treatment with Darco G-60 decolorizing carbon, the solution was concentrated to give a clear, colorless syrup of apibiose: $[\alpha]_{578}^{22}$ −69.1° (*c* 5, water). The material gave a single spot when examined by paper chromatography. Throughout this section chromatograms were

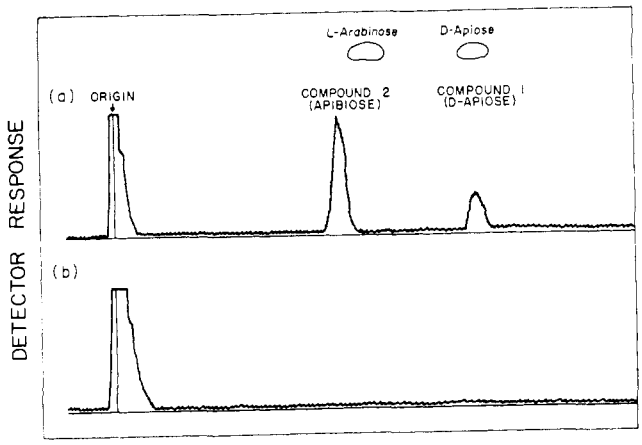

FIGURE 1: Radiochromatogram scans of heated and unheated ^{14}C 22° sodium chloride soluble fraction. The polysaccharide fraction was dissolved in water at a concentration of 3 mg/ml. The pH of the solution was 4.5. Aliquots (0.1 ml) of this solution were (a) heated at 100° for 3 hr or (b) kept at 22°. The assays were further treated as described in the Materials and Methods.

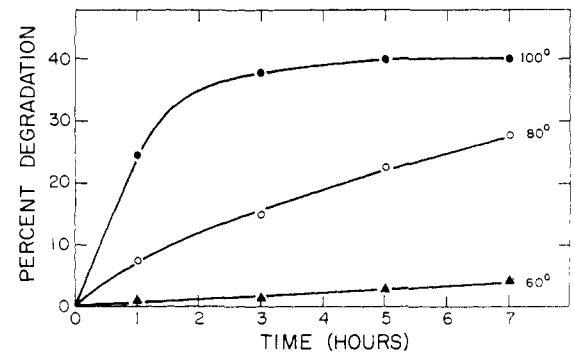

FIGURE 2: Degradation of sodium [^{14}C]apiogalacturonan IIa as a function of temperature and time. The polysaccharide was dissolved in water at a concentration of 3 mg/ml. The pH of the solution was 4.5. Aliquots (0.1 ml) of this solution were heated for 1, 3, 5, and 7 hr at 60° (▲), 80° (○), and 100° (●). The assays were further treated as described in the Materials and Methods.

developed in solvent A and the $AgNO_3$ method was used to visualize the sugars.

Sodium Borohydride Reduction of [^{14}C]Apibiose. [^{14}C]Apibiose was reduced with a tenfold molar excess of $NaBH_4$ in water at 37° for 16 hr. The solution was neutralized with acetic acid and then concentrated to dryness. Borate was removed as the methyl ester by distillation under reduced pressure. The reduced material was purified by paper chromatography, first in solvent A and then in solvent D. The material migrated as a single peak in the latter solvent and was named apibiitol.

Hydrolysis of [^{14}C]Apibiose, [^{14}C]Apibiitol, and [^{14}C]Apibiose Phenylosotriazole. [^{14}C]Apibiose, [^{14}C]apibiitol, and [^{14}C]apibiose phenylosotriazole were hydrolyzed with 0.1 N H_2SO_4 at 100° for 1 hr. Before [^{14}C]apibiose and [^{14}C]-apibiitol were hydrolyzed, nonradioactive D-apiose was added to the former and nonradioactive D-apiose and D-apiitol were added to the latter. The cooled solutions were neutralized with NaOH to pH 6. In a few experiments [^{14}C]-apibiose was hydrolyzed with potassium phosphate buffers without adding nonradioactive D-apiose. Hydrolysates were spotted directly on paper. The papers were developed in solvent A and scanned and the radioactive areas were cut out and counted in scintillation solution B. Greater than 90% of the starting radioactivity could be accounted for in the one or two radioactive areas obtained from each compound.

Preparation of Apibiose Phenylosotriazole. To a 2% (w/v) solution of apibiose in 5.0 M sodium acetate buffer, pH 4.5, at 80° was added a tenfold molar excess of phenylhydrazine hydrochloride in an equal volume of the same buffer at 80°. After the solution was heated for 1 hr at 90–95° water (3 volumes) was added. The resulting precipitate was allowed to settle for 2–3 hr at 4°. It was then collected by centrifugation at 35,000*g* for 20 min, dissolved in 1–3 ml of ethanol and reprecipitated by addition of 10 volumes of water. After 4 hr at 4° the amorphous apibiose phenylosazone was collected by centrifugation and dried *in vacuo* over P_2O_5: yield 75–80%, mp 91–93°.

To a 1.5% (w/v) suspension of apibiose phenylosazone in water heated to reflux was added a solution of 1.1 molar equiv of $CuSO_4 \cdot 5H_2O$ in 0.5 volume of water at 80°. The resulting reddish solution was refluxed for 1 hr. The solution at 4° was filtered through Whatman No. 5 paper, concentrated, and streaked on several sheets of unwashed paper. The papers were developed in solvent A and then examined with an ultraviolet lamp having a maximum emission at 366 nm. The fluorescing band at R_F 0.69 was eluted with water. The eluates were combined, concentrated, and continuously extracted with diethyl ether for 30–36 hr. The diethyl ether solution was treated with Darco G-60, filtered, and concentrated to incipient cloudiness. Colorless needles formed on standing at 22°: yield 25% from apibiose phenylosazone. The apibiose phenylosotriazole was recrystallized from diethyl ether by solution in water and reextraction with diethyl ether: mp 116.5–117.0°, $[\alpha]_{578}^{22}$ −80.0° (*c* 5, water).

Anal. Calcd for $C_{16}H_{21}N_3O_7$: C, 52.3; H, 5.8; N, 11.5. Found: C, 51.9; H, 5.8; N, 11.4.

Periodate Oxidation and Determination of Formaldehyde. Apibiose phenylosotriazole (4 to 17 mg) was 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 $NaIO_4$ in an equal volume of water was added. The solutions were kept in the dark for 2 hr at 22°. Trial experiments had shown that formaldehyde production was complete after 1 hr. The solutions were added to a column of Dowex 1-X8 (200–400 mesh, acetate form) 1.0 cm in diameter and 7.0 cm high. The formaldehyde quantitatively passed through the column and was determined with chromotropic acid (Speck, 1962). Chromotropic acid was recrystallized once from 50% (v/v) aqueous ethanol.

Isolation and Pectinase Hydrolysis of Partially Degraded Sodium [^{14}C]Apiogalacturonan IIa. Sodium [^{14}C]apiogalacturonan IIa was degraded following the procedure described in the above section on apibiose isolation. The precipitate obtained on addition of acetone to the heat-treated sodium [^{14}C]apiogalacturonan IIa was designated, partially degraded sodium [^{14}C]apiogalacturonan IIa. This precipitate was dissolved in water, concentrated, and lyophilized. This partially degraded polysaccharide (1 mg) was hydrolyzed with 2 mg of pectinase for 2 hr as described previously (Hart and Kindel, 1970). The hydrolysis was terminated by heating

TABLE I: The Change in the Ratio of Degradation Products Obtained from Sodium [^{14}C]Apiogalacturonan IIa As a Function of pH.[a]

pH	D-Apiose (%)	Apibiose (%)	Apibiose/ D-Apiose	Per Cent 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

[a] The quantities used and the procedures followed were the same as those described in the legend of Figure 3 except that the heating period was 180 min. Per cents are expressed as the per cent of the total recovered disintegrations per minute. Per cent degradation is defined in the Results.

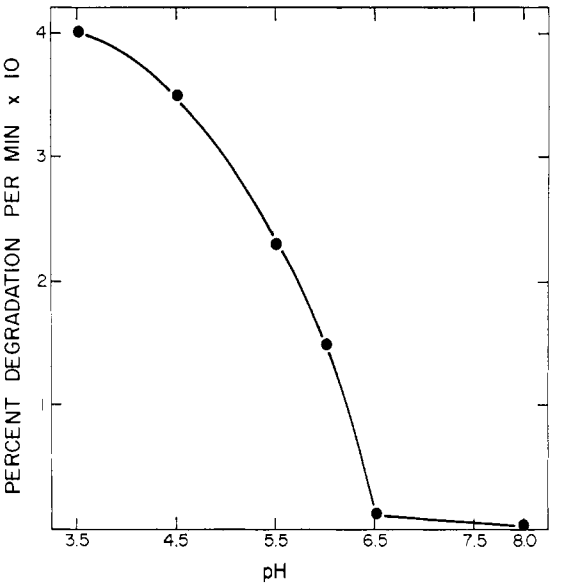

FIGURE 3: The rate of degradation of sodium [^{14}C]apiogalacturonan IIa as a function of pH. The polysaccharide was dissolved in 0.05 M potassium phosphate buffers, at pH 3.5, 4.5, 5.5, 6.0, 6.5, and 8.0, at a concentration of 3 mg/ml. Aliquots (0.1 ml) of each of the solutions were heated at 100° for 20, 40, and 60 min. The assays were further treated as described in the Materials and Methods. At each pH, a linear relationship was obtained over the entire 60-min period when per cent degradation was plotted *vs.* time. From the slopes of these lines were calculated the rates plotted in this Figure.

for 1 min at 100°. Denatured protein was removed by centrifugation and the supernatant solution was spotted directly on unwashed paper. The papers were developed in solvent D and scanned and the radioactive areas were cut out and counted in scintillation solution B. Per cent hydrolysis was the per cent of the total recovered disintegrations per minute in D-galacturonic acid.

Results

Degradation of [^{14}C]*Apiogalacturonans.* When the ^{14}C 22° sodium chloride soluble fraction isolated from *L. minor* cell wall material was heated in water at 100° and then chromatographed on paper in solvent A, two radioactive compounds of high R_F were detected (Figure 1). As described below these two compounds were identified as apibiose and D-apiose. The same two radioactive compounds were also obtained from the other three fractions obtained by ammonium oxalate extraction. No other radioactive compounds that migrated in this solvent were obtained from any of these fractions. Treatment of the 70° water fraction and the residue fraction in a similar manner did not yield these two radioactive compounds. The six cell wall fractions referred to here are those listed in Table 1 of Hart and Kindel (1970). For reasons given earlier, sodium [^{14}C]apiogalacturonan IIa was used to characterize the degradation reaction. In this paper per cent degradation is defined as the per cent of the total recovered disintegrations per minute in apibiose and D-apiose. By definition, the maximum per cent degradation that any apiogalacturonan can undergo is equal to its per cent D-apiose content. Where rates of the degradation reaction are mentioned these are initial rates. In our early degradation experiments unheated samples were used as controls. However this practice was discontinued when it was found that the per cent degradation in the controls was consistently zero.

The degradation reaction was dependent on the temperature and on the length of the heating period (Figure 2). Degradation at 100° was more than half-complete in 1 hr and reached a final value of 40% after 5 hr. The value of 40% corresponds well to the D-apiose content of this polysaccharide. There was still extensive degradation at 80°. However at 60°, the per cent degradation after 7 hr was only 10% of that which occurred at 100°.

The rate of the degradation reaction was also dependent on the pH of the solution (Figure 3). The rate declined steadily from pH 3.5 to almost zero at pH 6.5. The pH also affected the ratio of apibiose to D-apiose (Table I). As the pH of the solution was decreased from 5.5 to 3.5, there was a decrease in the ratio of apibiose to D-apiose. This latter result was obtained when the heating period was 180 min. Other heating periods were not tried.

The rate and limit of the degradation reaction were slightly inhibited in the presence of salt. These experiments and those illustrated in Figure 2 were carried out at the same time. Sodium [^{14}C]apiogalacturonan IIa was dissolved in 0.1 M and 0.2 M potassium phosphate buffers, both at pH 4.5, at a concentration of 3 mg/ml. The experiments were carried out at 100° in the same way as those described in the legend of Figure 2. The shape of the curves obtained when per cent degradation was plotted *vs.* time was the same for all three conditions; water (depicted in Figure 2) and the above two concentrations of potassium phosphate. The limit of the degradation obtained after 7 hr in 0.2 M buffer was 90% of that obtained in water. The rate of the degradation in 0.1 M and 0.2 M potassium phosphate buffer was 88 and 68%, respectively, of that obtained in water.

The rate of the degradation reaction and the ratio of apibiose to D-apiose was also a function of the concentration of apiogalacturonan. Sodium [^{14}C]apiogalacturonan IIa was dissolved in water at pH 4.5, at concentrations of 1, 5, and 10 mg per ml. Aliquots (0.1 ml) of each of the solutions

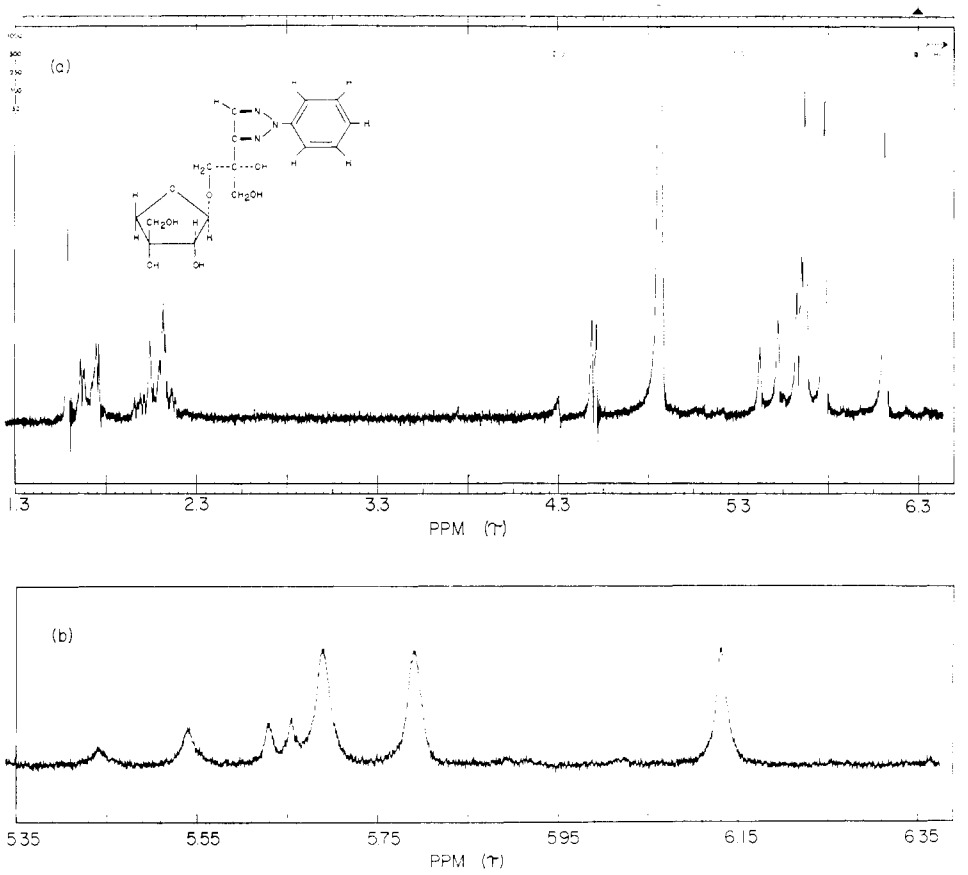

FIGURE 4: Proton magnetic resonance spectrum of apibiose phenylosotriazole in D_2O.

were heated at 100° for 20, 40, and 60 min. The assays were further treated as described in the Materials and Methods. The rates obtained at 1 and 5 mg per ml were 70 and 86%, respectively, of that obtained at 10 mg/ml. For all three concentrations a linear relationship was obtained over the entire 60-min period when per cent degradation was plotted *vs.* time. The ratio of apibiose to D-apiose decreased as the polysaccharide concentration was increased. After 90 min at 100°, the ratios were 10.4, 8.2, and 6.5 at 1, 5, and 10 mg per ml, respectively. An accurate measurement of per cent degradation could not be made with polysaccharide concentrations below 1 mg/ml and concentrations above 10 mg/ml were prohibitive because of the quantity of material needed.

When sodium [^{14}C]apiogalacturonan IIa, at a concentration of 3 mg/ml, was heated for 6 hr at 70° in 0.5% (w/v) ammonium oxalate, pH 6.2, there was 3.8% degradation.

Identification of Compound **1** *As* D-*Apiose.* Radioactive compound **1**, when chromatographed in solvents A to D, migrated as a single peak with the same R_F as D-apiose. When the chromatograms were sprayed with aniline hydrogen phthalate, a yellow spot developed which coincided with the radioactive peak and gave an intense white fluorescence in ultraviolet light (max 366 nm). These properties are characteristic of D-apiose.

Identification of Compound **2** *As a Disaccharide of* D-*Apiose (Apibiose).* Radioactive compound **2** chromatographed as a single peak in solvents A to D. It had an $R_{\text{D-apiose}}$ of 0.53, 0.65, 0.75, and 0.95 in solvents A, B, C, and D, respectively. When the chromatograms were sprayed with aniline hydrogen phthalate, a yellow spot developed which coincided with the radioactive peak and gave an intense white fluorescence in ultraviolet light (max 366 nm). On hydrolysis with 0.1 N sulfuric acid, radioactive compound **2** was converted quantitatively into a radioactive compound which chromatographed on paper with the same R_F as D-apiose. Nonradioactive compound **2** was hydrolyzed under the same conditions and the crystalline α-benzyl-α-phenylhydrazone derivative of the hydrolysis product was prepared: mp 137–138°, $[\alpha]_{546}^{22}$ −86.3° (2.7% in pyridine). The same derivative of D-apiose was prepared: mp 137–138°, $[\alpha]_{546}^{22}$ −90.1° (4.0% in pyridine). The melting point of a mixture of the two was not depressed. Acid hydrolysis of radioactive reduced compound **2** and the radioactive phenylosotriazole derivative of compound **2** resulted in the release of equimolar quantities (as determined by radioactivity measurements) of D-apiose and D-apiitol from the former and D-apiose and D-apiose phenylosotriazole from the latter. These data identified compound **2** as a disaccharide of D-apiose (apibiose).

Characterization of Apibiose. Two derivatives of apibiose were prepared, the amorphous phenylosazone and the crystalline phenylosotriazole.

Apibiose phenylosotriazole was oxidized with sodium metaperiodate and the amount of formaldehyde released was determined. In four experiments, 2.04, 2.06, 2.03, and 2.02 moles of formaldehyde were produced per mole of derivative.

The proton magnetic resonance spectrum of apibiose phenylosotriazole is shown in Figure 4a. There were no detectable signals between τ 0.0–1.5 and 6.3–10.0. Integration of the τ 1.5–2.2 region indicated that it contained six protons and that the singlet at τ 1.59 was due to a single proton. This singlet was assigned to the hydrogen attached to the osotriazole ring. The position of this singlet at low field strength, the sharpness of the peak, and the presence of a similar peak with the same chemical shift in the spectrum of D-apiose phenylosotriazole served as the basis for this assignment. The complex pattern at τ 1.62–2.20 is attributed to the five hydrogens attached to the benzene ring. Integration showed the peaks in the region from τ 4.3 to 6.3 contained the remaining 10 protons. This region contained a one-proton doublet ($J_{1,2}$ = 2.6 Hz) at τ 4.50, a large HOD peak at 4.87, a partially resolved area at 5.3–5.9, and a two-proton singlet at 6.11. The doublet at τ 4.50 was assigned to the anomeric hydrogen of the D-apiose component (Hall, 1964). Expansion of the partially resolved area (τ 5.3–5.9) gave the spectrum seen in Figure 4b. This area contained a two-proton AB quartet ($J \sim 10$ Hz) at approximately τ 5.6, a one-proton doublet ($J_{1,2}$ = 2.6 Hz) at 5.64, a two-proton singlet at 5.67, and a two-proton singlet at 5.78. Integration of peak areas showed that the two high-field absorption peaks of the AB quartet coincide with the singlets at τ 5.67 and 5.78. The doublet at 5.64 was assigned to the hydrogen on carbon atom 2 of D-apiose. The three two-proton singlets and the two-proton AB quartet can not be assigned with certainty. The small broad peak at τ 4.30 is a spinning side band of HOD.

[^{14}C]Apibiose was hydrolyzed with 0.05 M potassium phosphate buffer at pH 2.5, 3.5, and 4.5. The disaccharide was dissolved in each buffer at a concentration of 1 mg/ml. Aliquots (0.2 ml) of each of the solutions were heated at 100° for 30, 60, 90, and 180 min. The assays were treated as described in the Materials and Methods. Per cent hydrolysis of radioactive apibiose is the per cent of the total recovered disintegrations per minute in D-apiose. After 180 min at 100° the hydrolysis at pH 2.5 was 42% where as the hydrolysis at pH 3.5 and 4.5 was 3.7 and 2.4%, respectively. At each pH, a linear relationship was obtained over the entire 180-min period when per cent hydrolysis was plotted *vs.* time.

Characterization of Partially Degraded Sodium [^{14}C]Apiogalacturonan IIa. When partially degraded sodium [^{14}C]apiogalacturonan IIa was chromatographed on paper in solvent A, all of the radioactive material remained at the origin. On treatment with pectinase, 75% of the partially degraded sodium [^{14}C]apiogalacturonan IIa was converted into a radioactive compound having the same R_F as D-galacturonic acid in solvent D. Further characterization of the partially degraded polysaccharide was not attempted.

Discussion

A general characteristic of the group of apiogalacturonans previously isolated from *L. minor* (Hart and Kindel, 1970) is their low content of esterified D-galacturonic acid residues. The pH profile of the degradation reaction (Figure 3) and the low ester content of the representative apiogalacturonan used, lead us to conclude that the degradation reported here is a hydrolysis reaction rather than an elimination reaction of the type described for pectins by Albersheim (1959) and

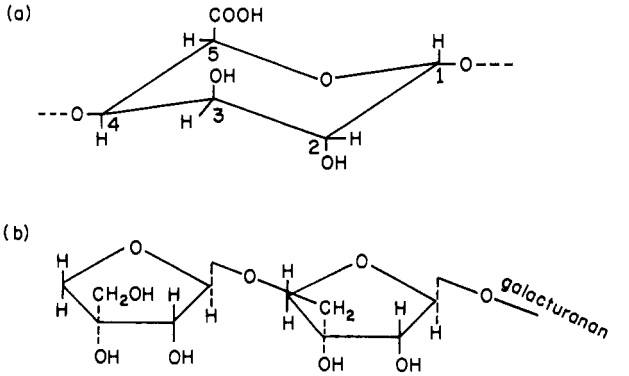

FIGURE 5: (a) Possible conformation of the D-galacturonic acid residues in apiogalacturonans. (b) Structure of the apibiose unit of apiogalacturonans. In b dotted lines indicate stereochemistry is unknown.

Albersheim *et al.* (1960). The failure to detect any unsaturated degradation products and the ready conversion of the partially degraded polysaccharide into D-galacturonic acid by pectinase treatment confirmed this conclusion. The unusually mild acid conditions needed for degradation indicated that it is not a simple acid hydrolysis. The stability of the glycosidic bond in apibiose to conditions which caused rapid cleavage of the glycosidic bond between apibiose and the galacturonan supports this suggestion. On the basis of these results we postulate that the degradation reaction is a hydrogen ion catalyzed hydrolysis in which there is transannular participation of the free carboxyl group of the D-galacturonic acid residues in the cleavage of the glycosidic bond between the apibiose side chains and the galacturonan. The increase in the rate of the degradation reaction and the decrease in the ratio of apibiose to D-apiose as the apiogalacturon concentration was increased indicated that some hydrolysis may be due to intermolecular interactions, particularly at higher concentrations of the polysaccharide. It would be of interest to determine if the polysaccharides isolated by Bouveng (1965) and by Aspinall and Baillie (1963) also undergo a degradation of the type reported here.

Apibiose can only be linked to the D-galacturonic acid residues at carbon atoms 2, or 3, or both since the galacturonan is linked α-(1→4). If apibiose was attached at carbon atom 3 and the D-galacturonic acid residues were in the conformation shown in Figure 5a, the glycosidic bond between apibiose and the galacturonan would be in close proximity to the carboxyl group. Alternatively, a conformation could be attained through heating which allowed the facile removal of apibiose when it was attached to carbon atom 2.

Results obtained in the present study and previously (Hart and Kindel, 1970) show that the D-apiose in the apiogalacturonans is almost entirely present as disaccharide side chains and that these side chains are all attached to the galacturonans in a similar manner. The failure to detect apibiose or D-apiose when the residue fraction was heated does not prove that the remaining 76% of the D-apiose of the cell wall is in a different type of polysaccharide. If carboxyl groups participate in the degradation reaction and if these are complexed with cations when the apiogalacturonans are in the solid state, the degradation reaction may not occur. At this time nothing may be stated about the

structure of the compounds containing D-apiose remaining in the residue fraction.

Previously, concern was expressed that the apiogalacturonans extracted by ammonium oxalate at 70° may be partially degraded (Hart and Kindel, 1970). The results show that any degradation of the type described in this paper was minor.

The information obtained from the hydrolysis of apibiose, from the reduction of apibiose and subsequent hydrolysis of apibiitol, from the specific rotation of the α-benzyl-α-phenylhydrazone derivative of the hydrolysis product of apibiose and from preparation of apibiose phenylosotriazole showed that apibiose is a reducing disaccharide of D-apiose and that the glycosidic linkage is between carbon atom 1 of the nonreducing D-apiose molecule and carbon atom 3 or 3′ of the other D-apiose. The previous periodate results obtained with intact apiogalacturonans (Hart and Kindel, 1970), confirmed this latter conclusion. The data obtained from the periodate oxidation of apibiose phenylosotriazole established that the glycosidic linkage between the two D-apiose molecules is 1→3′.

The structure assigned to apibiose phenylosotriazole is consistent with its proton magnetic resonance spectrum (Figure 4a, b). Coupling constant values have been used to establish the configuration of glycosidic bonds. Based on calculations made by Karplus (1959) and Conroy (1960), Rinehart *et al.* (1962) have stated that when a coupling constant equal to or less than 1 Hz is obtained the hydrogens on carbon atoms 1 and 2 of a nonrigid furanoside are *trans*. This statement has been found to be valid in at least one case (Jardetzky, 1962) and has been used to assign a configuration to the glycosidic linkage in two other cases (Rinehart *et al.*, 1962; McGilveray and Rinehart, 1965). It was also valid for a semirigid furanoside (Halford *et al.*, 1968). However, coupling constants for the *trans* hydrogens of β-ribofuranosyl nucleosides and nucleotides range from 2 to 7 Hz (Jardetzky, 1960, 1962). In addition, a coupling constant of 3.9 Hz has been obtained for the hydrogens on carbon atoms 1 and 2 of a nonrigid furanoside having the *cis* configuration (Hall, 1963). Results similar to those of Hall (1963) have been obtained for semirigid furanosides (Carey *et al.*, 1966; Ball *et al.*, 1969). Although the coupling constant value of 2.6 Hz indicates that H-1 and H-2 of the nonreducing D-apiose component are *trans* and therefore the configuration of the glycosidic linkage is β, an unequivocal assignment cannot be made.

The strong negative molecular rotations of apibiose ([*M*] = −195°) and apibiose phenylosotriazole ([*M*] = −294°) also suggest that the glycosidic linkage in apibiose is β (Klyne, 1950). However because the rotational contribution of the reducing D-apiose component of apibiose and the rotational contribution of carbon atom 3 of the D-apiose phenylosotriazole component of apibiose phenylosotriazole have not been evaluated, the rotational contribution of the methyl apioside component of each is not known with certainty. Proton magnetic resonance spectra and molecular rotations of model compounds are needed before the configuration of this glycosidic linkage can be conclusively assigned.

On the basis of the data available apibiose is *O*-β(?)-D-apio-(D or L)-furanosyl-(1→3′)-3-C-(hydroxymethyl)-*aldehydo*-D-*glycero*-tetrose. The disaccharide unit together with the α-D-galactopyranosyluronan is *O*-[*O*-β(?)-D-apio-(D or L)-furanosyl-(1→3′)-*O*-(α or β)-D-apio-(D or L)-furanosyl (1→2 or 3, or both)]-galacturonan (Figure 5b).

Beck (1967) postulated that the D-apiose in apiogalacturonans, isolated from *L. minor*, was present as monomeric side chains. However, his conclusion was based on hydrolysis data alone. A reexamination 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.

References

Albersheim, P. (1959), *Biochem. Biophys. Res. Commun. 1*, 253.

Albersheim, P., Neukom, H., and Deuel, H. (1960), *Arch. Biochem. Biophys. 90*, 46.

Aspinall, G. O., and Baillie, J. (1963), *J. Chem. Soc.*, 1702.

Ball, D. H., Carey, F. A., Klundt, I. L., and Long, L., Jr. (1969), *Carbohyd. Res. 10*, 121.

Beck, E. (1967), *Z. Pflanzenphysiol. 57*, 444.

Bouveng, H. O. (1965), *Acta Chem. Scand. 19*, 953.

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.

Carey, F. A., Ball, D. H., and Long, L., Jr. (1966), *Carbohyd. Res. 3*, 205.

Conroy, H. (1960), *in* Advances in Organic Chemistry. Methods and Results, Vol. 2, Raphael, R. A., Taylor, E. C., and Wynberg, H., Ed., New York, N. Y., Interscience, p 265.

Halford, N. H., Ball, D. H., and Long, L., Jr. (1968), *Carbohyd. Res. 8*, 363.

Hall, L. D. (1963), *Chem. Ind. (London)*, 950.

Hall, L. D. (1964), *Advan. Carbohyd. Chem. 19*, 51.

Hart, D. A., and Kindel, P. K. (1970), *Biochem. J. 116*, 569.

Jardetzky, C. D. (1960), *J. Amer. Chem. Soc. 82*, 229.

Jardetzky, C. D. (1962), *J. Amer. Chem. Soc. 84*, 62.

Karplus, M. (1959), *J. Chem. Phys. 30*, 11.

Kindel, P. K. (1970), *Carbohyd. Res.* (in press).

Klyne, W. (1950), *Biochem. J. 47*, XLI.

McGilveray, I. J., and Rinehart, K. L., Jr. (1965), *J. Amer. Chem. Soc. 87*, 4003.

Neal, D. L., and Kindel, P. K. (1970), *J. Bacteriol. 101*, 910.

Norris, L., Norris, R. E., and Calvin, M. (1955), *J. Exp. Bot. 6*, 64.

Partridge, S. M. (1949), *Nature 164*, 443.

Rinehart, K. L., Jr., Chilton, W. S., Hichens, M., and von Phillipsborn, W. (1962), *J. Amer. Chem. Soc. 84*, 3216.

Schmidt, O. Th. (1930), *Justus Liebigs Ann. Chem. 483*, 115.

Speck, J. C., Jr. (1962), *Methods Carbohyd. Chem. 1*, 441.

Trevelyan, W. E., Procter, D. P., and Harrison, J. S. (1950), *Nature 166*, 444.

Whistler, R. L., and BeMiller, J. N. (1962), *Methods Carbohyd. Chem. 1*, 47.