Synthesis, Characterization and Properties of Uridine 5'-(α -D-Apio-D-furanosyl Pyrophosphate)

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1. A method was developed for synthesizing UDP-apiose [uridine 5'-(α -D-apio-D-furanosyl pyrophosphate)] from UDP-glucuronic acid [uridine 5'-(α -D-glucopyranosyluronic acid pyrophosphate)] in 62% yield with the enzyme UDP-glucuronic acid cyclase. 2. UDP-apiose had the same mobility as uridine 5'-(α -D-xylopyranosyl pyrophosphate) when chromatographed on paper and when subjected to paper electrophoresis at pH 5.8. When [^3H]UDP-[U- ^{14}C]glucuronic acid was used as the substrate for UDP-glucuronic acid cyclase, the $^3\text{H}/^{14}\text{C}$ ratio in the reaction product was that expected if D-apiose remained attached to the uridine. In separate experiments doubly labelled reaction product was: (a) hydrolysed at pH 2 and 100°C for 15 min; (b) degraded at pH 8.0 and 100°C for 3 min; (c) used as a substrate in the enzymic synthesis of [^{14}C]apiin. In each type of experiment the reaction products were isolated and identified and were found to be those expected if [^3H]UDP-[U- ^{14}C]apiose was the starting compound. 3. Chemical characterization established that the product containing D-[U- ^{14}C]apiose and phosphate formed on alkaline degradation of UDP-[U- ^{14}C]apiose was α -D-[U- ^{14}C]apio-D-furanosyl 1:2-cyclic phosphate. 4. Chemical characterization also established that the product containing D-[U- ^{14}C]apiose and phosphate formed on acid hydrolysis of α -D-[U- ^{14}C]apio-D-furanosyl 1:2-cyclic phosphate was D-[U- ^{14}C]apiose 2-phosphate. 5. The half-life periods for the degradation of UDP-[U- ^{14}C]apiose to α -D-[U- ^{14}C]apio-D-furanosyl 1:2-cyclic phosphate and UMP at pH 8.0 and 80°C, at pH 8.0 and 25°C and at pH 8.0 and 4°C were 31.6s, 97.2 min and 16.5 h respectively. The half-life period for the hydrolysis of UDP-[U- ^{14}C]apiose to D-[U- ^{14}C]apiose and UDP at pH 3.0 and 40°C was 4.67 min. After 20 days at pH 6.2-6.6 and 4°C, 17% of the starting UDP-[U- ^{14}C]apiose was degraded to α -D-[U- ^{14}C]apio-D-furanosyl 1:2-cyclic phosphate and UMP and 23% was hydrolysed to D-[U- ^{14}C]apiose and UDP. After 120 days at pH 6.4 and -20°C 2% of the starting UDP-[U- ^{14}C]apiose was degraded and 4% was hydrolysed.

The biosynthesis of D-apiose (3-C-hydroxymethyl-aldehydo-D-glycero-tetrose) in cell-free systems from *Lemna* (duckweed) and *Petroselinum* (parsley) has been reported to occur by conversion of UDP-glucuronic acid [uridine 5'-(α -D-glucopyranosyluronic acid pyrophosphate)] into a product tentatively identified as UDP-apiose [uridine 5'-(α -D-apio-D-furanosyl pyrophosphate)] (Sandermann *et al.*, 1968; Sandermann & Grisebach, 1970). A complete characterization of the reaction product and a procedure for its preparation have not been described. The enzyme catalysing the conversion of UDP-glucuronic acid into the reaction product has been purified about 55-fold from *Lemna minor* by a modification of the procedure of Gustine (1969) and has been given the common name UDP-glucuronate cyclase (Kindel *et al.*, 1971). By using purified UDP-glucuronate cyclase we have developed a method for preparing the re-

action product in good yield and of sufficiently high purity to permit its complete characterization. The method of preparation, the characterization of the reaction product and its identification as UDP-apiose, and some properties of UDP-apiose, are described in the present paper.

In an earlier study on the biosynthesis of D-apiose from UDP-glucuronic acid in a cell-free system from *L. minor* we found that, when the assay mixtures were incubated, subjected to a brief heat-treatment and chromatographed, all under slightly alkaline conditions, a single compound containing D-apiose was formed, which proved to be neither UDP-apiose, a D-apiose phosphate monoester nor free D-apiose (Gustine & Kindel, 1969). The identification of this compound as α -D-apio-D-furanosyl 1:2-cyclic phosphate and its non-enzymic formation from UDP-apiose under exceedingly mild conditions are also described in the present paper. The formation of this sugar cyclic phosphate under conditions similar to those reported by Gustine & Kindel (1969) has also

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(A)

(B)

(C)

been observed by Sandermann & Grisebach (1970) and Mendicino & Hanna (1970). The structural formulae of UDP-apiose (A), α -D-apio-D-furanosyl 1:2-cyclic phosphate (B), and D-apiose 2-phosphate (C) are given. The configuration at C-3 of the D-apiose moiety in UDP-apiose and in α -D-apio-D-furanosyl 1:2-cyclic phosphate is assumed to be D as in apiin (Hulyalkar *et al.*, 1965). Since a sugar 1:2-cyclic phosphate is formed from UDP-apiose the configuration of UDP-apiose and α -D-apio-D-furanosyl 1:2-cyclic phosphate at C-1 must be α (Khorana *et al.*, 1957).

A preliminary account of some of this work has been presented (Kindel *et al.*, 1970).

Materials and Methods

Materials

UDP-glucuronic acid, UDP-xylose, UMP, UDP, UTP and NAD⁺ were obtained from P-L Biochemicals Inc., Milwaukee, Wis., U.S.A. Uridine 2':3'-cyclic phosphate was a gift from Dr. F. M. Rottman of this Department. 6-Benzylaminopurine and casein hydrolysate (enzymic) were purchased from ICN Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Polyethylenimine-600 was a gift from Dow Chemical Co., Midland, Mich., U.S.A. D-Apiose was isolated as described previously (Neal &

Kindel, 1970). Once-recrystallized 'apiin', isolated from *Petroselinum crispum* (parsley) seeds by the method of Gupta & Seshadri (1952), was used to prepare '7-apigenin β -D-glucopyranoside'. The conditions for hydrolysis of 'apiin' were the same as those described for the isolation of D-apiose from 'apiin' (Neal & Kindel, 1970). The '7-apigenin β -D-glucopyranoside' was collected by suction-filtration, washed thoroughly with water and recrystallized from aq. 95% (v/v) ethanol. The inverted commas indicate that 'apiin', and the '7-apigenin β -D-glucopyranoside' derived from it, were mixtures, presumably of closely related glycosides differing only slightly in their aglycone portions (Neal & Kindel, 1970). Use of these two names without inverted commas indicates the pure compounds. UDP-[U-¹⁴C]glucuronic acid (239 mCi/mmol) and the dipotassium salt of α -D-[U-¹⁴C]glucopyranosyl 1-phosphate (194 mCi/mmol) were obtained from New England Nuclear Corp., Boston, Mass., U.S.A. The tetralithium salt of [5-³H]UTP (12.1 Ci/mmol) was purchased from Schwarz/Mann, Orangeburg, N.Y., U.S.A. Crystalline calf liver UDP-glucose pyrophosphorylase (EC 2.7.7.9) (93 units/mg of protein) was a gift from Dr. J. A. Boezi of this Department. One unit of enzyme will form 1 μ mol of α -D-glucopyranosyl 1-phosphate (NADPH)/min at 25°C and pH 7.8 (Albrecht *et al.*, 1966). Crystalline yeast inorganic pyrophosphatase (EC 3.6.1.1) type II (approx. 250 units/mg of protein)

was purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. One unit of enzyme will liberate 1 μ mol of P_i /min at 25°C and pH 7.2. Partially purified calf liver UDP-glucose dehydrogenase (EC 1.1.1.22) was a gift from Dr. R. G. Hansen of Utah State University, Logan, Utah, U.S.A. One unit of enzyme will oxidize 1 μ mol of UDP-glucose/min at 25°C and pH 8.7. Purified *Escherichia coli* alkaline phosphatase (EC 3.1.3.1) (code BAPC; 35 units/mg of protein) was purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A. One unit of enzyme will liberate 1 μ mol of *p*-nitrophenol/min at 25°C.

Methods

General methods. α -D-[U- 14 C]Apio-D-furanosyl 1:2-cyclic phosphate and D-[U- 14 C]apiose 2-phosphate were eluted from paper chromatograms with water at 4°C and were stored at -20°C. Electrophoresis was carried out with unwashed Whatman no. 1 paper. The following buffers were employed: A, 0.2M-ammonium acetate, pH 5.8; B, 0.2M-ammonium acetate, pH 4.5; C, 0.05M-Tris, pH 7.8, prepared from Tris and HCl. At pH 5.8 and 4.5 electrophoresis was performed at 30 V/cm and 4°C with a Pherograph Original Frankfurt type 64 apparatus (distributed by Brinkmann Instruments Inc., Westbury, N.Y., U.S.A.). At pH 7.8 it was performed at 15 V/cm and 22°C with a Beckman paper-electrophoresis apparatus, model R (Beckman Instruments Inc., Palo Alto, Calif., U.S.A.). Radioactivity was detected on chromatograms with a Packard radiochromatogram scanner, model 7201 (Packard Instrument Co. Inc., Downers Grove, Ill., U.S.A.). All other radioactivity measurements were made with a Packard Tri-Carb liquid-scintillation counter, model 3310, by using one of the following scintillation solutions: (A) Bray (1960); (B) 2,5-bis-(5-*t*-butylbenzoxazol-2-yl)thiophen in reagent-grade toluene (4g/litre). With scintillation solution (B) the radioactive compounds were counted directly on paper by cutting out the appropriate portions of the chromatogram and completely immersing them in this solution. The counting efficiencies with scintillation solutions (A) and (B) were 78 and 63% respectively. Total phosphate was determined by Bartlett's (1959) modification of the Fiske & SubbaRow (1925) method with KH_2PO_4 as the standard. KH_2PO_4 was heated at 100°C for 18h and stored in a desiccator over $CaCl_2$ before use. Blank determinations were made on eluates obtained by eluting papers that were identical in size with those containing the samples but were cut from blank chromatograms. Blank and sample chromatograms were developed identically.

Paper chromatography. Descending paper chromatography and either washed or unwashed Whatman no. 3MM paper were used. Washing was with 0.1M-citric acid followed by water. Chromatography was

at 22°C unless otherwise stated. Unwashed Whatman no. 3MM paper impregnated with neutralized 2.5% (v/v) polyethylenimine-600 was used with solvent (H) (Verachtert *et al.*, 1965). The following solvents were employed: (A) aq. 95% (v/v) ethanol-1.0M-ammonium acetate, pH 7.5 (7:3, v/v); (B) propan-1-ol-ethyl acetate-water (7:1:2, by vol.); (C) aq. 75% (v/v) ethanol; (D) aq. 95% (v/v) ethanol-1.0M-ammonium acetate, pH 7.5 (9:1, v/v); (E) propan-1-ol-ethyl acetate-1.0M-potassium phosphate, pH 6.0 (7:1:4, by vol.); (F) aq. 95% (v/v) ethanol-1.0M-ammonium acetate, pH 3.8 (9:1, v/v); (G) aq. 95% (v/v) ethanol-1.0M-ammonium acetate, pH 3.8 (7.5:2.5, v/v); (H) 0.3M-LiCl; (I) ethyl acetate-water-acetic acid-formic acid (18:4:3:1, by vol.); (J) butan-1-ol-acetic acid-water (4:1:5, by vol., upper phase). When using solvents (F) and (G) equilibration of the papers was for 5 min or less and was with a solution of ethanol and water (Tovey & Roberts, 1970). Compounds were detected on chromatograms as follows: sugars with aniline hydrogen phthalate (Partridge, 1949); α -D-glucopyranosyl 1-phosphate by the procedure of Bandurski & Axelrod (1951); compounds absorbing u.v. light with an appropriate lamp.

Culture of *L. minor*. *L. minor*, obtained from the Red Cedar River at East Lansing, Mich., U.S.A., and freed from other organisms by using the procedure described by Hillman (1961), was grown on modified medium V of Norris *et al.* (1955). One litre of medium contained: KNO_3 , 2.02g; KH_2PO_4 , 0.272g; $MgSO_4 \cdot 7H_2O$, 0.493g; $Ca(NO_3)_2 \cdot 4H_2O$, 0.236g; NaCl, 0.01g; 1ml of iron solution containing 0.02g of $FeSO_4 \cdot 7H_2O$; H_3BO_3 , 2.86mg; $MnSO_4 \cdot H_2O$, 1.54mg; $CuSO_4 \cdot 5H_2O$, 0.58mg; $Na_2MoO_4 \cdot 2H_2O$, 0.118mg; $ZnCl_2$, 0.105mg; 2.5ml of an aqueous solution containing 0.025mg of 6-benzylaminopurine. The iron solution was prepared with EDTA as described by Jacobson (1951). The pH of the medium was adjusted to 6.0 with aq. NH_3 . Distilled water was used throughout. This medium was designated the inorganic medium. *L. minor*, grown aseptically on inorganic medium in 1-litre Erlenmeyer flasks, was used to inoculate small plastic pans (29cm $\times$ 33cm) containing inorganic medium where growth was non-aseptic. The plants were harvested when the fronds completely covered the surface of the medium (approx. 10-12 days). When larger quantities of *L. minor* were needed, plants grown for 10 days in one small plastic pan were transferred to a large circular plastic pan (76cm internal diam.) and were allowed to grow an additional 7 days. Each large pan yielded approx. 150g wet wt. of *L. minor*. The plants were illuminated continuously from the top by incandescent and fluorescent lights, which provided approx. 2700 lx at the upper surface of the fronds. The temperature was 26-29°C. Inorganic medium supplemented with 0.2% D-glucose and 0.06% casein hydrolysate was used to prepare and maintain sterile

cultures of *L. minor*. This medium was designated the organic medium.

Preparation and assay of UDP-glucuronic acid cyclase and apiin synthase. UDP-glucuronic acid cyclase was purified from fresh *L. minor*, which had been grown on the inorganic medium as described above. The purification and assay procedures were modifications of those described by Gustine (1969). As a routine 300g wet wt. of *L. minor* was processed. Potassium phosphate and 3-mercaptopropane-1,2-diol were used throughout. The crude cell-free extract was treated with protamine sulphate before the $(\text{NH}_4)_2\text{SO}_4$ step was performed. The precipitate obtained when the $(\text{NH}_4)_2\text{SO}_4$ concentration at 0°C was increased from 50% to 61% of saturation was used in the Sephadex G-100 step. In the DEAE-Sephadex step a linear gradient of KCl was used that increased from 0.1 to 0.3M. The appropriate fractions from the DEAE-Sephadex column were concentrated with a Diaflo cell and a UM-10 membrane (Amicon Corp., Lexington, Mass., U.S.A.). The final concentrations of potassium phosphate, EDTA, 3-mercaptopropane-1,2-diol, NAD^+ and UDP-[U- ^{14}C]glucuronic acid in the standard assay mixture were 0.05M, 0.1mM, 80mM, 1.0mM and 0.106mM respectively. The standard assay mixture was incubated for 5min at 25°C and then was heated for 3min at 100°C. Paper chromatography of the assay mixture was with modified solvent (H) (the LiCl concentration was 0.2M). Apiin synthase was purified from *P. crispum* leaves (Watson, 1971). One unit of each enzyme is the amount that forms 1 μmol of either UDP-apiose or apiin/min.

Preparation of [^3H]UDP-[U- ^{14}C]glucuronic acid. [^3H]UDP-[U- ^{14}C]glucose was prepared from [^3H]UTP and $\alpha\text{-D}$ -[U- ^{14}C]glucopyranosyl 1-phosphate (Fitzgerald & Ebner, 1966) and was converted into [^3H]UDP-[U- ^{14}C]glucuronic acid (Castanera & Hassid, 1965). The incubation mixture contained 1.4nmol of [^3H]UTP (16.9 μCi), 10.3nmol of $\alpha\text{-D}$ -[U- ^{14}C]glucopyranosyl 1-phosphate (2.0 μCi), 7nmol of UTP, 1.8 μmol of magnesium acetate, 25 μmol of glycine-NaOH buffer, pH 8.7, 5.4 units of UDP-glucose pyrophosphorylase and 1.2 units of inorganic pyrophosphatase in a volume of 0.48ml. The mixture was incubated for 30min at 25°C and then 3nmol of UTP and 2.7 units of UDP-glucose pyrophosphorylase were added, making the final volume 0.5ml. The mixture was incubated for an additional 30min at 25°C. To the solution was added 0.4 μmol of NAD^+ and 0.05 unit of UDP-glucose dehydrogenase, and after incubation for 1h at 25°C the mixture was heated for 1min at 100°C. Portions of the preparation were examined by paper chromatography with solvents (A) and (H). Only [^3H]UDP-[U- ^{14}C]glucuronic acid and a small amount of D-[U- ^{14}C]glucose were observed. The latter compound had been present in the $\alpha\text{-D}$ -[U- ^{14}C]glucopyranosyl 1-phosphate. It did not

contaminate the final product, [^3H]UDP-[U- ^{14}C]apiose, since it was separated from the UDP-pentoses during paper chromatography with solvent (E). Neither radioactive substrate contaminated the [^3H]UDP-[U- ^{14}C]glucuronic acid, since both were made limiting at different stages in the procedure.

Preparation of $\alpha\text{-D}$ -[U- ^{14}C]apio-D-furanosyl 1:2-cyclic phosphate. To obtain a high radiochemical yield the following procedure was used. A reaction mixture, basically identical with the reaction mixture described in the Results section for the synthesis of UDP-[^{14}C]apiose, was incubated for 1h at 25°C and then heated at 100°C for 3min. The solution and two washes with aq. 50% (v/v) ethanol were streaked on unwashed paper and the paper was developed with solvent (A). Scanning showed that three radioactive compounds were present. In an earlier paper these were designated compounds I, II and III (Gustine & Kindel, 1969). Compounds I and II were previously identified as UDP-[U- ^{14}C]glucuronic acid and UDP-[U- ^{14}C]xylose and in the present paper compound III is identified as $\alpha\text{-D}$ -[U- ^{14}C]apio-D-furanosyl 1:2-cyclic phosphate. The use of more enzyme and a longer incubation time than before resulted in almost quantitative utilization of UDP-[U- ^{14}C]glucuronic acid. The small amount of D-[U- ^{14}C]apiose occasionally present had an R_f value slightly greater than that of $\alpha\text{-D}$ -[U- ^{14}C]apio-D-furanosyl 1:2-cyclic phosphate in solvent (A). The yields of $\alpha\text{-D}$ -[U- ^{14}C]apio-D-furanosyl 1:2-cyclic phosphate ranged from 70 to 75%. The values were adjusted for the loss of C-6 of the glucuronic acid moiety as $^{14}\text{CO}_2$.

With certain modifications the same procedure as that described above was used to obtain a maximum number of μmoles of the sugar cyclic phosphate. The components of the reaction mixture and their concentrations were the same except that the starting concentration of UDP-[U- ^{14}C]glucuronic acid was 0.5mM, the enzyme concentration was 6.3–6.5 munits/ml of reaction mixture and the incubation time was 3h. The final volume of the reaction mixture ranged from 10 to 30ml. The following additional steps were used in the treatment of the heated reaction mixture. Salts were removed from the solution as it was being concentrated by several ethanol precipitations followed by cooling the suspensions to 4°C. Each salt precipitate was washed twice with aq. 95% (v/v) ethanol. Yields of $\alpha\text{-D}$ -[U- ^{14}C]apio-D-furanosyl 1:2-cyclic phosphate ranged from 61 to 67%.

The $\alpha\text{-D}$ -[U- ^{14}C]apio-D-furanosyl 1:2-cyclic phosphate obtained by both procedures was rechromatographed on washed paper, first in solvent (B) and then in solvent (C). In a typical preparation of high-specific-radioactivity $\alpha\text{-D}$ -[U- ^{14}C]apio-D-furanosyl 1:2-cyclic phosphate, chromatography with solvent (B) showed that the radioactivity in D-[U- ^{14}C]apiose 2-phosphate and D-[U- ^{14}C]apiose was 0.7 and 0.5% respectively of that present in $\alpha\text{-D}$ -[U- ^{14}C]apio-D-

furanosyl 1:2-cyclic phosphate. After chromatography of the product in solvent (C) a single peak was obtained on scanning. α -D-[U- 14 C]Apio-D-furanosyl 1:2-cyclic phosphate prepared in this manner is homogeneous (Gustine & Kindel, 1969).

Preparation of D-[U- 14 C]apiose 2-phosphate. D-[U- 14 C]Apiose 2-phosphate was prepared by heating α -D-[U- 14 C]apio-D-furanosyl 1:2-cyclic phosphate in 0.01M-HCl (the pH was established with a micro-electrode) at 100°C for 5 min. The hydrolysate was neutralized to pH 7–8 with NaOH, streaked on unwashed paper and the paper was developed in solvent (D). D-[U- 14 C]Apiose 2-phosphate was eluted and rechromatographed on washed paper, first in solvent (B) and then in solvent (C). In solvents (B) and (C) a single peak was obtained on scanning.

Chromatography of α -D-[U- 14 C]apio-D-furanosyl 1:2-cyclic phosphate and D-[U- 14 C]apiose 2-phosphate in solvents (B) and (C). These two compounds normally migrated with higher R_F values when rechromatographed on washed paper in solvents (B) or (C). However they did not migrate with characteristic higher R_F values but rather a range of R_F values was obtained when the different preparations of each compound were rechromatographed. This was probably due to the various amounts of salts removed from these compounds during the first chromatography in solvents (B) and (C).

Hydrolysis of α -D-[U- 14 C]apio-D-furanosyl 1:2-cyclic phosphate at pH 2.0 and 100°C (Fig. 1). To tubes at 25°C were added α -D-[U- 14 C]apio-D-furanosyl 1:2-cyclic phosphate and then sufficient HCl to give a final pH of 2.0. Each sample solution contained 27.2 nmol of α -D-[U- 14 C]apio-D-furanosyl 1:2-cyclic phosphate (4776 d.p.m.) and had a final volume of 0.09 ml. The total radioactivity in a sample solution was 4846 d.p.m. as measured with scintillation solution (A). The solutions were heated at 100°C for the times indicated (Fig. 1). After incubation, each solution was cooled to 4°C and adjusted to pH 7–8 with 0.08M-NaOH. The neutralized solutions and the zero-time sample (control) were kept at 4°C until the incubations were completed and then the solutions and two water washes were applied to washed paper. For the zero-time sample, the HCl and NaOH were combined first and then this solution was added to the solution containing α -D-[U- 14 C]apio-D-furanosyl 1:2-cyclic phosphate. The proper quantities of HCl and NaOH required were predetermined by using a micro-electrode and a solution of α -D-[U- 14 C]apio-D-furanosyl 1:2-cyclic phosphate identical with that used in the experiment. The papers were developed with solvent (D), scanned, and the appropriate radioactive areas were cut out and counted in scintillation solution (B). The amount of α -D-[U- 14 C]apio-D-furanosyl 1:2-cyclic phosphate (4846 d.p.m. minus the zero-time sample values) in each sample solution was set equal to 100%. All samples were corrected

for the D-[U- 14 C]apiose 2-phosphate (57 d.p.m.) and D-[U- 14 C]apiose (13 d.p.m.) present in the zero-time sample. The percentage of the total radioactivity used (4846 d.p.m.) that was recovered in the three compounds measured at each time-period ranged from 95 to 101% and averaged 99%.

Hydrolysis of D-[U- 14 C]apiose 2-phosphate with 0.125M-H $_2$ SO $_4$ at 100°C (Fig. 2). To tubes at 25°C were added D-[U- 14 C]apiose 2-phosphate, 1.0 μ mol of D-apiose and then 0.5M-H $_2$ SO $_4$ to give a final concentration of 0.125M-H $_2$ SO $_4$. Each sample solution contained 38.0 nmol of D-[U- 14 C]apiose 2-phosphate (6674 d.p.m.) and had a final volume of 0.1 ml. The total radioactivity in a sample solution was 6803 d.p.m. as measured with scintillation solution (A). The solutions were heated at 100°C for the times indicated (Fig. 2). The rest of the procedure was the same as that described for the acid hydrolysis of α -D-[U- 14 C]apio-D-furanosyl 1:2-cyclic phosphate except that 0.5M-NaOH was used for neutralization and the papers were developed with solvent (A). The amount of D-[U- 14 C]apiose 2-phosphate (6803 d.p.m. minus the zero-time sample value) in each sample solution was set equal to 100%. All samples were corrected for the D-[U- 14 C]apiose (129 d.p.m.) present in the zero-time sample. The percentage of the total radioactivity used (6803 d.p.m.) that was recovered in the two compounds measured at each time-period ranged from 92 to 104% and averaged 99%.

Degradation of UDP-[U- 14 C]apiose at pH 8.0 and 80°C (Fig. 3). To tubes at 25°C, each containing potassium phosphate solution, was added UDP-[U- 14 C]apiose at 4°C. The potassium phosphate solution was prepared as described below. In the typical experiment described each sample solution contained 18.4 pmol of UDP-[U- 14 C]apiose (8079 d.p.m.), 7.92 pmol of UDP-[U- 14 C]xylose (3487 d.p.m.) and 0.44 pmol of UDP-[U- 14 C]glucuronic acid (230 d.p.m.) and had a final phosphate concentration of 0.05M, a final pH of 8.0 and a final volume of 0.08 ml. The total radioactivity in a sample solution was 13071 d.p.m. as measured with scintillation solution (A). The solutions were incubated at 80°C for the times indicated (Fig. 3). After incubation, each solution was immediately cooled to 4°C and adjusted to pH 6.5–6.8 with 0.12M-HCl. The neutralized solutions and the zero-time sample (control) were kept at 4°C until the incubations were completed (total time for the incubations was approx. 20 min) and then the solutions and two washes with aq. 50% (v/v) ethanol were applied to washed paper at 4°C, care being taken that the paper did not become dry. For the zero-time sample, the potassium phosphate solution and HCl were combined first and then to this solution was added the solution containing the UDP-[U- 14 C]apiose. Without equilibration the paper was developed at 4°C with solvent (G)

for 18h, scanned, and the appropriate radioactive areas were cut out and counted in scintillation solution (B). Percentage degradation is the percentage of the initial UDP-[U-¹⁴C]apiose that was converted into α -D-[U-¹⁴C]apio-D-furanosyl 1:2-cyclic phosphate and UMP. The amount of UDP-[U-¹⁴C]apiose (9354 d.p.m. minus the zero-time sample value) initially present in each sample solution was set equal to 100%. All samples were corrected for the D-[U-¹⁴C]apiose and α -D-[U-¹⁴C]apio-D-furanosyl 1:2-cyclic phosphate (total of 1275 d.p.m.) present in the zero-time sample. The percentage of the total radioactivity used (13 071 d.p.m.) that was recovered in the two sugar nucleotides, D-[U-¹⁴C]apiose and α -D-[U-¹⁴C]apio-D-furanosyl 1:2-cyclic phosphate ranged from 93 to 101% and averaged 96%. The results from three separate experiments are plotted. Separation of D-[U-¹⁴C]apiose and α -D-[U-¹⁴C]apio-D-furanosyl 1:2-cyclic phosphate was only partial and they were assayed for radioactivity together. Separation of these two compounds was not necessary, since the amount of D-[U-¹⁴C]apiose initially present will not change under the conditions of the experiment. The potassium phosphate solution was prepared by mixing K₂HPO₄ with potassium phosphate buffer, pH 8.0. The amount of K₂HPO₄ used was such that when the solution containing the UDP-[U-¹⁴C]apiose was mixed with the potassium phosphate solution the final pH of the sample solution was 8.0. Different UDP-[U-¹⁴C]apiose preparations required different amounts of K₂HPO₄. The proper quantities of K₂HPO₄ and HCl required were predetermined by using a micro-electrode and a solution of UDP-[U-¹⁴C]apiose identical with that used in the experiment.

Hydrolysis of UDP-[U-¹⁴C]apiose at pH 3.0 and 40°C (Fig. 4). To tubes at 25°C were added UDP-[U-¹⁴C]apiose at 4°C and then sufficient HCl to give a final pH of 3.0. In the typical experiment described each sample solution contained 18.9 pmol of UDP-[U-¹⁴C]apiose (8322 d.p.m.), 8.61 pmol of UDP-[U-¹⁴C]xylose (3789 d.p.m.), 0.40 pmol of UDP-[U-¹⁴C]glucuronic acid (211 d.p.m.) and had a final volume of 0.06 ml. The total radioactivity in a sample solution was 13856 d.p.m. as measured with scintillation solution (A). The solutions were incubated at 40°C for the times indicated (Fig. 4). After incubation, each solution was immediately cooled to 4°C and adjusted to pH 5.5–6.5 with aq. 0.04 M-NH₃. The neutralized solutions and the zero-time sample (control) were kept at 4°C until the incubations were completed (total time for the incubations was approx. 25 min) and then the solutions and two washes with aq. 50% (v/v) ethanol were applied to washed paper at 4°C, care being taken that the paper did not become dry. For the zero-time sample, the HCl and aq. NH₃ were combined first and then this solution was added to the solution containing the UDP-

[U-¹⁴C]apiose. The proper quantities of HCl and aq. NH₃ required were predetermined by using a micro-electrode and a solution of UDP-[U-¹⁴C]apiose identical with that used in the experiment. Without equilibration the paper was developed at 4°C with solvent (F) for 18h, scanned, and the appropriate radioactive areas were cut out and counted in scintillation solution (B). Percentage hydrolysis is the percentage of the initial UDP-[U-¹⁴C]apiose that was converted into D-[U-¹⁴C]apiose and UDP. The amount of UDP-[U-¹⁴C]apiose (9856 d.p.m. minus the zero-time sample values) initially present in each sample solution was set equal to 100%. All samples were corrected for the D-[U-¹⁴C]apiose (903 d.p.m.) and α -D-[U-¹⁴C]apio-D-furanosyl 1:2-cyclic phosphate (631 d.p.m.) present in the zero-time sample. The percentage of the total radioactivity used (13856 d.p.m.) that was recovered in the two sugar nucleotides, D-[U-¹⁴C]apiose and α -D-[U-¹⁴C]apio-D-furanosyl 1:2-cyclic phosphate ranged from 91 to 100% and averaged 97%. The results from three separate experiments are plotted. Within experimental error the amount of α -D-[U-¹⁴C]apio-D-furanosyl 1:2-cyclic phosphate isolated from each solution, including the zero-time sample, was the same, indicating that this compound was not hydrolysed by the above conditions.

Calculation of kinetic constants. Pseudo-first-order rate constants (*k*) and the energy of activation (*E_a*) were calculated from slopes obtained from the data by using the computer program POLFIT of ACTS Computing Corp., Southfield, Mich., U.S.A. (Wampler, 1969). This program performs a least-squares analysis by the method of orthogonal polynomials. The following relationships were used: (A) $-k = 2.303 \times \text{slope}$; (B) half-life = $0.693/k$; (C) $-E_a = 2.303 \times 8.314 \times \text{slope } J^\circ K^{-1} \cdot \text{mol}^{-1}$.

Results

Synthesis of the product of the reaction catalysed by UDP-glucuronate cyclase

The assay procedure initially used to detect UDP-glucuronate cyclase involved incubating the enzyme with UDP-[U-¹⁴C]glucuronic acid and NAD⁺ in sodium phosphate buffer, pH 8.0, for 5 min at 25°C, heating the assay mixture briefly at 100°C and then chromatographing the mixture in solvent (A) (Gustine & Kindel, 1969). When this procedure was used the D-[U-¹⁴C]apiose synthesized was found in compound III. We subsequently found that if the assay mixture was not heated at 100°C but cooled to 4°C, and spotted on unwashed paper and chromatographed in solvent (A) at this same temperature, little compound III was obtained. Instead, a D-[U-¹⁴C]apiose-containing compound with an *R_F* value of UDP-xylose was obtained. This new compound

was quantitatively converted into compound III when heated briefly at 100°C in 0.05 M-sodium phosphate buffer, pH 8.0. These results showed that the true product of the reaction was not compound III but was a compound that migrated with an R_F value of UDP-xylose. A method for preparing the reaction product enzymically from UDP-[U-¹⁴C]-glucuronic acid was developed and is described.

A reaction mixture containing 2.51 nmol of UDP-[U-¹⁴C]glucuronic acid (0.6 μ Ci), 0.6 μ mol of NAD⁺, 30 μ mol of potassium phosphate buffer, pH 8.0, 0.06 μ mol of EDTA (disodium salt), 48 μ mol of 3-mercaptopropane-1,2-diol and 2.31 munits of UDP-glucuronate cyclase [0.206 mg; the enzyme was stored at -20°C in 50% (v/v) glycerol and just before use was dialysed against 0.05 M-potassium phosphate buffer, pH 8.0, containing 0.1 mM-EDTA (disodium salt) and 0.08 M-3-mercaptopropane-1,2-diol and then was assayed at the same time the reaction product was synthesized], purified through the DEAE-Sephadex step, in a total volume of 0.6 ml was prepared. The mixture was incubated 15 min at 25°C, cooled to 4°C, and then was immediately acidified to pH 6.0-6.5 with acetic acid. The remaining steps were carried out at 4°C. The reaction mixture and two washes with aq. 50% (v/v) ethanol were streaked on washed paper over 8 cm, care being taken that the paper did not become dry. The paper was immediately developed in solvent (E) for 20 h. Without having been dried, the chromatogram was cut and the radioactive reaction product was located by scanning a thin strip of the chromatogram. The reaction product was eluted from the remainder of the chromatogram with 1.0 ml of 1 mM-potassium phosphate, pH 6.0. The pH of the eluate was 6.5. If the pH of the eluate is not between 6.2 and 6.6 the pH of the potassium phosphate solution used for eluting should be changed. The paper wick used for this elution was prepared from washed Whatman no. 1 paper. The washing procedure was the same as that used for the Whatman no. 3MM paper. By measuring the amount of radioactivity in the eluate, the yield of reaction product plus UDP-[U-¹⁴C]xylose and a small amount of UDP-[U-¹⁴C]glucuronic acid in this typical preparation was determined to be 89%. If formed, both α -D-[U-¹⁴C]apio-D-furanosyl 1:2-cyclic phosphate and D-[U-¹⁴C]apiose were well separated from the reaction product but not from each other by solvent (E). The proportion of the original radioactivity present in these two compounds was 1.5%. Most of any unchanged UDP-[U-¹⁴C]glucuronic acid was also separated from the reaction product by solvent (E). Of the original radioactivity, 4.9% migrated as UDP-[U-¹⁴C]glucuronic acid. Where necessary, percentages were adjusted for the loss of C-6 of the glucuronic acid moiety as ¹⁴CO₂. A portion of the eluate containing the reaction product was adjusted to pH 8.0 with a potassium phosphate solution (for

procedure see the Materials and Methods section), heated for 3 min at 100°C, and the degradation products were chromatographed on unwashed paper in solvent (A). The size of the portion was such that UDP-[U-¹⁴C]glucuronic acid could be detected if 1% or more of the total radioactivity in the portion was in this compound. Radioactivity was measured with scintillation solution (B) and the proportions in α -D-[U-¹⁴C]apio-D-furanosyl 1:2-cyclic phosphate, UDP-[U-¹⁴C]xylose and UDP-[U-¹⁴C]glucuronic acid were found to be 70, 28.4 and 1.6% respectively. The amount of UDP-[U-¹⁴C]glucuronic acid was too small to affect the results of experiments involving the labelled reaction product. Since 94% of the radioactivity used was recovered in these three compounds we assumed that the reaction product, UDP-[U-¹⁴C]xylose, and UDP-[U-¹⁴C]glucuronic acid were the only radioactive compounds in the eluate. Therefore the yield of reaction product was 62%. A very small amount of the reaction product in the eluate was in a degraded state. The degradation occurred during elution at 4°C and was detected by rechromatographing a portion of the eluted reaction product in solvent (F). Of the total radioactivity recovered after chromatography in solvent (F), 2.1% migrated with R_F of α -D-[U-¹⁴C]apio-D-furanosyl 1:2-cyclic phosphate. This showed that 3.0% of the reaction product had been degraded to UMP and α -D-[U-¹⁴C]apio-D-furanosyl 1:2-cyclic phosphate. Of the total radioactivity used for rechromatography, 99% was recovered in the reaction product, UDP-[U-¹⁴C]xylose, UDP-[U-¹⁴C]glucuronic acid and α -D-[U-¹⁴C]apio-D-furanosyl 1:2-cyclic phosphate. Solvent (E) separated most or all of the NAD⁺ from the reaction product. If reaction product free of salts is needed, solvent (E) can be modified by replacing the 1 mM-potassium phosphate, pH 6.0, with water. The reaction product was stored in the eluting solution at pH 6.2-6.6. Storage was at 0°C or -20°C. When stored at -20°C the eluate was made 35% (v/v) with respect to ethanol. Reaction product stored with ethanol was used in the identification experiments. Reaction product stored in phosphate buffer containing no ethanol was used in the acid-hydrolysis and alkaline-degradation experiments. In this paper reaction product refers to the D-[U-¹⁴C]apiose-containing product of the reaction catalysed by UDP-glucuronate cyclase.

Identification of the reaction product

The reaction product was chromatographed on washed paper at 4°C in a modified solvent (G), in which the ratio of aq. 95% (v/v) ethanol-1.0 M-ammonium acetate, pH 3.8, was 13:7 (v/v). In this solvent, the reaction product had the same R_F value as UDP-xylose and gave a single peak on scanning

Table 1. *Products obtained on acid hydrolysis of doubly labelled reaction product and their $^3\text{H}/^{14}\text{C}$ ratios*

The experiment was performed and the products were identified as described in the Results section. Small amounts of [^3H]UMP and α -D-[U- ^{14}C]apio-D-furanosyl 1:2-cyclic phosphate were present with the reaction product before the experiment was performed (see the Results section). The values were not corrected for this [^3H]UMP and for the D-[U- ^{14}C]apirose derived from the α -D-[U- ^{14}C]apio-D-furanosyl 1:2-cyclic phosphate.

Compound	$^3\text{H}/^{14}\text{C}$ ratio	^3H (%)	^{14}C (%)
Doubly labelled reaction product*	10.6	100	100
Products from acid hydrolysis			
UDP	910	85.9	1.0
UMP	109	10.3	1.0
D-Apirose-D-xylose	0.01	0.1	95.0
Total		96.3	97.0

* For preparation see the Results section. The starting percentages of ^3H and ^{14}C are based on the the radioactivity present in both the reaction product and [^3H]UDP-[U- ^{14}C]xylose.

except for the small peak due to α -D-[U- ^{14}C]apio-D-furanosyl 1:2-cyclic phosphate. When subjected to paper electrophoresis at pH 5.8, the mobility of the reaction product was the same as that of UDP-xylose.

Reaction product was prepared from [^3H]UDP-[U- ^{14}C]glucuronic acid and was characterized by basically the same procedures as those described above. The yield of reaction product and the characterization results were essentially the same as those given above. A portion of the [^3H]UDP-[U- ^{14}C]glucuronic acid was chromatographed on washed paper in solvent (B) to separate it from the contaminating D-[U- ^{14}C]glucose (see the Materials and Methods section). The $^3\text{H}/^{14}\text{C}$ ratios in [^3H]UDP-[U- ^{14}C]glucuronic acid and in reaction product plus [^3H]UDP-[U- ^{14}C]xylose were 8.91 and 10.6 respectively. These are the results expected if the D-[U- ^{14}C]apirose remained attached to the [^3H]uridine and one carbon atom of the D-glucuronic acid moiety was lost as $^{14}\text{CO}_2$.

A portion of the doubly labelled reaction product (0.08 nmol) was heated in 0.01 M-HCl for 15 min at 100°C. The hydrolysis mixture, UDP, UMP, D-xylose and D-apiose, was chromatographed on washed paper in solvent (G) at 4°C and the paper was scanned. The four radioactive products obtained were eluted from the chromatogram with water at 4°C. Chromatography in solvent (G) and co-chromatography with authentic compounds in solvents (A) and (H) identified two products as [^3H]UDP and [^3H]UMP. Of the two [^3H]UDP predominated. The other two products were only partially resolved by solvent (G) and were eluted together. Chromatography in solvent (G) and co-chromatography on washed paper in solvent (I) with authentic compounds showed that these two products were D-[U- ^{14}C]xylose and D-[U- ^{14}C]apirose. The radioisotope content and $^3\text{H}/^{14}\text{C}$ ratio of each product were determined (Table 1). The small amount of α -D-

[U- ^{14}C]apio-D-furanosyl 1:2-cyclic phosphate present with the reaction product before the experiment was performed was hydrolysed to D-[U- ^{14}C]apirose and D-[U- ^{14}C]apirose 2-phosphate. However, the quantity of D-[U- ^{14}C]apirose 2-phosphate expected was too small to be detected. Formation of D-[U- ^{14}C]apirose, D-[U- ^{14}C]xylose or [^3H]UMP was not observed in a control where the reaction product (0.08 nmol) was kept at pH 6.5 and 4°C for the duration of the experiment and then was treated as described above. Presumably [^3H]UDP was also not formed in the control, although this could not be established since UDP, UDP-xylose and the reaction product have similar R_f values in solvent (G). These results established that the products of the acid hydrolysis of the reaction product were [^3H]UDP and D-[U- ^{14}C]apirose.

A second portion of the doubly labelled reaction product (0.08 nmol) was heated in 0.05 M-potassium phosphate, pH 8.0 (pH adjusted as described in the Materials and Methods section), for 3 min at 100°C. The degradation mixture, UMP and UDP-xylose were chromatographed on washed paper in solvent (A) at 22°C for 48 h and the paper was scanned. Three radioactive compounds were obtained. One migrated off the paper and was collected and the other two were eluted from the chromatogram with water at 4°C. Chromatography in solvent (A) and co-chromatography with authentic compounds in solvent (H) identified the compounds remaining on the paper as [^3H]UMP and [^3H]UDP-[U- ^{14}C]xylose. Chromatography of the third compound on washed paper, first in solvent (D) and then in solvent (B) followed by hydrolysis of this compound with 0.125 M- H_2SO_4 for 2 h at 100°C and treatment of the hydrolysate as described by Gustine & Kindel (1969) for compound III, identified it as α -D-[U- ^{14}C]apio-D-furanosyl 1:2-cyclic phosphate. The radioisotope content and $^3\text{H}/^{14}\text{C}$ ratio of each compound were determined (Table

Table 2. *Products obtained on alkaline degradation of doubly labelled reaction product and their $^3\text{H}/^{14}\text{C}$ ratios*

The experiment was performed and the compounds were identified as described in the Results section. The values were not corrected for the small amounts of [^3H]UMP and α -D-[U- ^{14}C]apio-D-furanosyl 1:2-cyclic phosphate present with the reaction product before the experiment was performed (see the Results section).

Compound	$^3\text{H}/^{14}\text{C}$ ratio	^3H (%)	^{14}C (%)
Doubly labelled reaction product*	10.6	100	100
Products from alkaline degradation			
UMP	418.0	61.6	1.5
α -D-Apio-D-furanosyl 1:2-cyclic phosphate	0.01	0.1	57.7
Other compound			
UDP-xylose	10.2	35.4	36.2
Total		97.1	95.4

* See footnote of Table 1.

2). There was no detectable alkaline degradation of [^3H]UDP-[U- ^{14}C]xylose as evidenced by its complete recovery and by the absence of D-[U- ^{14}C]xylose after acid hydrolysis of the material with the R_F value of α -D-[U- ^{14}C]apio-D-furanosyl 1:2-cyclic phosphate. Formation of α -D-[U- ^{14}C]apio-D-furanosyl 1:2-cyclic phosphate or [^3H]UMP was not observed in the control. The control was the same as that described in the preceding paragraph. These results established that the products from the alkaline degradation of the reaction product were [^3H]UMP and α -D-[U- ^{14}C]apio-D-furanosyl 1:2-cyclic phosphate. They also showed that [^3H]UDP-[U- ^{14}C]xylose was not degraded under these conditions.

The reaction product acted as a D-[U- ^{14}C]apiosyl donor. A reaction mixture was prepared that contained 0.055 nmol of doubly labelled reaction product, 15 nmol of '7-apigenin β -D-glucopyranoside', 7.5 μmol of potassium phosphate buffer, pH 8.0, and 0.4 munit of apiin synthase (0.208 mg; apiin synthase was stored and dialysed as described above for UDP-glucuronate cyclase and was assayed at the same time as the experiment was performed) purified through the DEAE-Sephadex step of Watson (1971) in 0.15 ml. The mixture was incubated for 5 min at 25°C and then heated for 3 min at 100°C. The reaction mixture, UDP, UMP, UDP-xylose, α -D-[U- ^{14}C]apio-D-furanosyl 1:2-cyclic phosphate and 'apiin' were chromatographed on washed paper in solvent (A) at 22°C and the paper was scanned. The radioactive compounds were eluted with aq. 75% (v/v) ethanol at 4°C. Two radioactive compounds were obtained that were not obtained in the controls. Chromatography in solvent (A) and co-chromatography in solvent (H) identified one compound as [^3H]UDP. The second compound chromatographed in solvent (A) and co-chromatographed on washed

paper in solvent (J) with apiin (the major component in 'apiin'). Hydrolysis of this compound with 0.05 M- H_2SO_4 for 1 h at 100°C in the presence of D-apiose and treatment of the hydrolysate as described by Gustine & Kindel (1969) for compound III yielded D-[U- ^{14}C]apiose. These results established that [^3H]UDP and [^{14}C]apiin are the products of the reaction catalysed by apiin synthase. We have previously shown that two spots were obtained after 'apiin' was chromatographed on washed paper in solvent (J) (Neal & Kindel, 1970). However, in the above experiment, only [^{14}C]apiin was observed after chromatography in solvent (J). This does not necessarily mean that D-[U- ^{14}C]apiose was transferred only to 7-apigenin β -D-glucopyranoside. It is possible that D-[U- ^{14}C]apiose was also transferred to the other acceptor(s) present in the '7-apigenin β -D-glucopyranoside' but in amounts too small to be detected. This was not investigated. It should be noted that in solvent (A) 'apiin' migrated as a single component whereas in solvent (J) the same quantity of the same 'apiin' separated into two components. Three other compounds were also present on the original chromatogram. Two of these were not widely separated and were eluted together. Chromatography in solvent (A) and co-chromatography in solvent (H) identified them as [^3H]UMP and [^3H]UDP-[U- ^{14}C]xylose. The third compound was α -D-[U- ^{14}C]apio-D-furanosyl 1:2-cyclic phosphate. The identification procedure for this compound was the same as that described in the preceding paragraph. Alkaline degradation of unchanged doubly labelled reaction product accounts for the formation of the latter compound and [^3H]UMP. The radioisotope content and $^3\text{H}/^{14}\text{C}$ ratio of each compound were determined (Table 3). The following three control experiments were performed: (1) with heated apiin synthase; (2) with heated reaction product; (3) with both heated apiin synthase

Table 3. *Products obtained on incubation of doubly labelled reaction product and 7 -apigenin β -D-glucopyranoside' with apiin synthase and their $^3\text{H}/^{14}\text{C}$ ratios*

The experiment was performed and the compounds were identified as described in the Results section. The values were not corrected for the small amounts of [^3H]UMP and α -D-[U- ^{14}C]apio-D-furanosyl 1:2-cyclic phosphate present with the reaction product before the experiment was performed (see the Results section).

Compound	$^3\text{H}/^{14}\text{C}$ ratio	^3H (%)	^{14}C (%)
Doubly labelled reaction product*	10.6	100	100
Products			
Apiin	0.1	0.4	54.8
UDP	5512	50.2	0.1
Other compounds			
α -D-Apio-D-furanosyl 1:2-cyclic phosphate	0.9	1.4	14.9
UMP plus UDP-xylose	16.7	44.1	28.3
Total		96.1	98.1

* See footnote of Table 1.

and heated reaction product. The reaction mixtures for the controls were identical with that described above except that apiin synthase or doubly labelled reaction product or both were heated for 3 min at 100°C and pH 8.0 before being added, and authentic 'apiin' and UDP were added to serve as markers. The treatment of the control reaction mixtures and the determination of the percentage of each radioisotope in a compound were also the same as described above. The starting quantities of ^3H and ^{14}C were each assigned a value of 100% and are based on the radioactivity present in both the reaction product and [^3H]UDP-[U- ^{14}C]xylose. The ^{14}C and ^3H contents of the following compounds from the control experiments were: apiin (^3H , 0.6%, average of 0.0, 1.3, 0.4%; ^{14}C , 2.1%, average of 2.3, 1.6, 2.5%); UDP (^3H , 3.9%, average of 2.1, 7.2, 2.3%; ^{14}C , 0.5%, average of 0.0, 0.1, 1.4%); α -D-apio-D-furanosyl 1:2-cyclic phosphate (^3H , 2.0%, average of 2.0, 3.0, 1.1%; ^{14}C , 57.0%, average of 51.9, 67.4, 51.7%); UMP plus UDP-xylose (^3H , 89.5%, average of 97.4, 82.0, 89.1%; ^{14}C , 35.4%, average of 39.2, 30.3, 36.8%). The three numbers used to calculate each average value refer, in order, to the values obtained from control experiments 1, 2 and 3 respectively.

The above results, together with those in the following two sections, identify the product of the reaction catalysed by UDP-glucuronate cyclase as UDP-apiose.

Identification of α -D-[U- ^{14}C]apio-D-furanosyl 1:2-cyclic phosphate (compound III)

In a previous paper (Gustine & Kindel, 1969), which described the formation of D-[U- ^{14}C]apiose

from UDP-[U- ^{14}C]glucuronic acid in a cell-free system from *L. minor*, we detected only one compound containing D-[U- ^{14}C]apiose and established that this compound was not UDP-apiose, a D-apiose phosphate monoester or D-apiose. Although the compound was incompletely characterized at the time and therefore designated compound III, the following information about it had been obtained: (1) it released D-[U- ^{14}C]apiose when hydrolysed in strong acid; (2) it contained phosphate, but the phosphate was not released on treatment with *E. coli* alkaline phosphatase; (3) when hydrolysed in weak acid it was converted into a D-[U- ^{14}C]apiose phosphate monoester; (4) it contained no uridine.

The following additional information on compound III has now been obtained. It had an average D-[U- ^{14}C]apiose/total phosphate ratio of 1:1.02. Ratios from three experiments were 1:1.08, 1:0.95 and 1:1.02. D-[U- ^{14}C]apiose content was determined by measuring radioactivity. Electrophoresis showed that compound III migrated as an anion at pH 4.5 and 7.8. Its relative mobility with respect to uridine 2':3'-cyclic phosphate was approximately the same at both pH 4.5 and 7.8 (Table 4). In contrast, the relative mobilities of both UMP and D-[U- ^{14}C]apiose 2-phosphate increased with respect to uridine 2':3'-cyclic phosphate when the pH was changed from 4.5 to 7.8 (Table 4). This showed that the phosphate group in compound III had only one ionizable hydrogen atom. Compound III was rapidly hydrolysed at pH 2.0 and 100°C to D-[U- ^{14}C]apiose 2-phosphate, D-[U- ^{14}C]apiose (Gustine & Kindel, 1969) and presumably P_i (Fig. 1). Reaction of compound III with sodium metaperiodate resulted in the release of [^{14}C]formaldehyde. Compound III (24230 d.p.m.; 0.138 μmol), 415.7 μmol of form-

Table 4. *Electrophoresis of compound III and D-[U-¹⁴C]apiose 2-phosphate*

The experimental procedure is described in the Materials and Methods section. Values are the average of two separate experiments at each pH value. The mobility of uridine 2':3'-cyclic phosphate was set equal to 1.00.

Compound	Relative mobility	
	pH 4.5	pH 7.8
Uridine 2':3'-cyclic phosphate	1.00	1.00
UMP	0.88	1.16
Compound III	1.25	1.18
D-[U- ¹⁴ C]Apiose 2-phosphate	1.16	1.53

aldehyde and 87.5 μ mol of sodium metaperiodate in 1.0ml of water were kept in the dark for 10min at 22°C and then 3.0ml of water was added. The solution was applied to a column (0.86cm internal diam. $\times$ 7.5cm) of Dowex 1 (X8; 200–400 mesh; acetate form), and then the column was washed with water. Preliminary experiments established that formaldehyde passed quantitatively through such a column. Of the original radioactivity in compound III, 20.1% was eluted from the column with water. Preparation of the dimethone derivative identified this material as [¹⁴C]formaldehyde. The specific radioactivity of the derivative initially and after one recrystallization was 11400 d.p.m./mmol and 11600 d.p.m./mmol respectively. This established that one of the five carbon atoms of the D-[U-¹⁴C]apiose moiety of compound III was released as [¹⁴C]formaldehyde. The above results, when considered together with the origin and conditions for formation of compound III, identified compound III as α -D-[U-¹⁴C]apio-D-furanosyl 1:2-cyclic phosphate.

As was described above, compound III was formed during alkaline degradation of a compound, which, by a number of criteria other than its behaviour in alkaline solution, was identified as UDP-[U-¹⁴C]-apiose. It is known that sugar nucleotides with the proper stereochemistry at C-1 and C-2 of the sugar portion of the sugar nucleotide are degraded under alkaline conditions to yield the corresponding nucleoside 5'-monophosphate and sugar cyclic phosphate (Neufeld & Hassid, 1963). The identification of compound III as α -D-[U-¹⁴C]apio-D-furanosyl 1:2-cyclic phosphate is in accord with this fact.

Identification of D-[U-¹⁴C]apiose 2-phosphate

Acid hydrolysis of α -D-[U-¹⁴C]apio-D-furanosyl 1:2-cyclic phosphate resulted in the formation of D-[U-¹⁴C]apiose and a second hydrolysis product of unknown structure. The unknown product migrat-

Fig. 1. *Hydrolysis of α -D-[U-¹⁴C]apio-D-furanosyl 1:2-cyclic phosphate at pH 2.0 and 100°C*

The experimental procedure is described in the Materials and Methods section. The compounds measured were: $\circ$, α -D-[U-¹⁴C]apio-D-furanosyl 1:2-cyclic phosphate; Δ , D-[U-¹⁴C]apiose 2-phosphate; $\square$, D-[U-¹⁴C]apiose.

ed as an anion during electrophoresis at pH 4.5 and 7.8. The relative mobilities of the product and UMP with respect to uridine 2':3'-cyclic phosphate were greater at pH 7.8 than at 4.5 (Table 4). This showed that the phosphate group in the unknown product had two ionizable hydrogen atoms. Treatment of the product with *E. coli* alkaline phosphatase resulted in the release of D-[U-¹⁴C]apiose. When enzyme was omitted from the assay mixture D-[U-¹⁴C]apiose was not formed. The assay mixtures were chromatographed in solvent (A). The electrophoresis and phosphatase results indicated that the hydrolysis product was a D-[U-¹⁴C]apiose phosphate monoester. The acid-hydrolysis results (Fig. 2) showed that the D-[U-¹⁴C]apiose phosphate monoester was not an α -D-[U-¹⁴C]apiose 1-phosphate (Mendicino & Hanna, 1970). Reaction of the unknown D-[U-¹⁴C]-apiose phosphate with sodium metaperiodate resulted in the release of [¹⁴C]formaldehyde. The procedure for oxidation of D-[U-¹⁴C]apiose phosphate (15030 d.p.m.; 0.0856 μ mol) and the quantities of reagents used were the same as for α -D-[U-¹⁴C]apio-D-furanosyl 1:2-cyclic phosphate. The material eluted from the column contained 40.4% of the radioactivity originally present in the D-[U-¹⁴C]apiose phosphate and was identified as [¹⁴C]formaldehyde by preparation of the dimethone derivative. The specific radioactivity of the derivative initially and after one recrystallization was 14500 d.p.m./mmol and 14700 d.p.m./mmol respectively. These results established that two of the five carbon atoms of the D-[U-¹⁴C]apiose moiety of the unknown D-[U-¹⁴C]-apiose phosphate were released as [¹⁴C]formaldehyde.

Fig. 2. Hydrolysis of D-[U-¹⁴C]apiose 2-phosphate with 0.125M-H₂SO₄ at 100°C

The experimental procedure is described in the Materials and Methods section. The compounds measured were: Δ , D-[U-¹⁴C]apiose 2-phosphate; $\square$, D-[U-¹⁴C]apiose.

The only D-apirose phosphate that could release this amount of formaldehyde is D-apirose 2-phosphate. The other D-apirose phosphates either would release no formaldehyde or would release 1 mol of formaldehyde/mol of D-apirose phosphate. The above results identified the second acid-hydrolysis product as D-[U-¹⁴C]apiose 2-phosphate.

Stability of UDP-[U-¹⁴C]apiose

Degradation of UDP-[U-¹⁴C]apiose to α -D-[U-¹⁴C]apio-D-furanosyl 1:2-cyclic phosphate and UMP occurred rapidly at pH 8.0 and 80°C (Fig. 3). Kinetic constants are given in Table 5. There was no detectable degradation of UDP-[U-¹⁴C]xylose to α -D-[U-¹⁴C]xylopyranosyl 1:2-cyclic phosphate and UMP under these conditions, even during the longest heating periods. This was established by hydrolysing with acid the compounds with R_F values of α -D-[U-¹⁴C]apio-D-furanosyl 1:2-cyclic phosphate and UDP-[U-¹⁴C]xylose from reaction mixtures that were prepared and treated in the same way as those for Fig. 3 but were heated for 3 min at 100°C. From the former only D-[U-¹⁴C]apiose was obtained and from the latter only D-[U-¹⁴C]xylose was obtained.

The experiments described in this paragraph were basically identical with those illustrated in Fig. 3 except for the differences noted in each case. Degradation of UDP-[U-¹⁴C]apiose to α -D-[U-¹⁴C]apio-D-furanosyl 1:2-cyclic phosphate and UMP occurred at a substantial rate at pH 8.0 and 25°C. The sample solutions were incubated at 25°C for 0, 40, 80 or 120 min or for 0, 60, 120 or 180 min. Incubations were started such that all finished at the same time. Five separate experiments were carried out and the

results were graphed together. A straight line was obtained when log(percentage of UDP-[U-¹⁴C]apiose remaining) was plotted against time. Kinetic constants are given in Table 5. Degradation of UDP-[U-¹⁴C]apiose to these same two compounds also occurred at pH 8.0 and 4°C. In these experiments the test tubes containing the potassium phosphate solution were at 4°C and the sample solutions were incubated at 4°C for 0, 5, 8, 15 or 20 h. Incubations were started such that all finished at the same time. Three separate experiments were carried out and the results were graphed together. A straight line was obtained when log(percentage of UDP-[U-¹⁴C]apiose remaining) was plotted against time. Kinetic constants are given in Table 5. In these experiments at 25°C and 4°C, the percentage of the total radioactivity used that was recovered in the two sugar nucleotides, D-[U-¹⁴C]apiose and α -D-[U-¹⁴C]apio-D-furanosyl 1:2-cyclic phosphate ranged from 92 to 99% and 93 to 98% respectively, and for both averaged 96%. An Arrhenius plot was made with the data of Table 5 obtained at pH 8.0 (see insert, Fig. 3). An E_a of 81.2 kJ·mol⁻¹ was calculated for the alkaline degradation of UDP-[U-¹⁴C]apiose to UMP and α -D-[U-¹⁴C]apio-D-furanosyl 1:2-cyclic phosphate.

Breakdown of UDP-[U-¹⁴C]apiose occurred even at pH 6.2–6.6 and 4°C. Three separate experiments were performed. Portions of newly synthesized UDP-[U-¹⁴C]apiose were set aside and kept at 4°C. The pH of the solutions ranged from 6.2 to 6.6. The experiments were done in the same way as those illustrated in Fig. 3 except for the following differences. The potassium phosphate solution was not used and the sample solutions did not require neutralization. At 0, 7, 14 and 21 days duplicate portions were removed from a preparation, spotted on paper and chromatographed, all at 4°C. The papers were developed with solvent (F). After 20 days 17% (16, 18 and 18%) of the UDP-[U-¹⁴C]apiose was degraded to α -D-[U-¹⁴C]apio-D-furanosyl 1:2-cyclic phosphate and UMP and 23% (18, 23 and 29%) was hydrolysed to D-[U-¹⁴C]apiose and UDP for a total breakdown of 40%. The average and individual values for the three separate experiments are given before and within the parentheses respectively. The percentage of the total radioactivity used that was recovered in the two sugar nucleotides, D-[U-¹⁴C]apiose and α -D-[U-¹⁴C]apio-D-furanosyl 1:2-cyclic phosphate ranged from 95 to 101% and averaged 97%. The stability of UDP-[U-¹⁴C]apiose during storage at pH 6.4 and –20°C was examined. An ethanol–1 mM-potassium phosphate, pH 6.4 (7:13, v/v), solution containing UDP-[U-¹⁴C]apiose was stored at –20°C and duplicate portions were removed monthly. The remainder of the experiment was done in the same way as the experiments described above that were performed at pH 6.2–6.6 and 4°C. After

Fig. 3. Degradation of UDP-[U- 14 C]apiose to α -D-[U- 14 C]apio-D-furanosyl 1:2-cyclic phosphate and UMP at pH 8.0 and 80°C

The experimental procedure is described in the Materials and Methods section. The Arrhenius plot in the insert was made as described in the Results section and E_a was calculated from the formula given in the Materials and Methods section.

Table 5. Kinetic constants for the degradation and hydrolysis of UDP-[U- 14 C]apiose

The values were obtained as described in the Materials and Methods and in the Results sections.

Temp. (°C)	pH	Pseudo-first-order rate constant (k)	Half-life
80	8.0	0.0219 s^{-1}	31.6 s
25	8.0	0.00713 min^{-1}	97.2 min
4	8.0	0.0421 h^{-1}	16.5 h
40	3.0	0.149 min^{-1}	4.67 min

120 days 2% of the UDP-[U- 14 C]apiose was degraded to α -D-[U- 14 C]apio-D-furanosyl 1:2-cyclic phosphate and UMP and 4% was hydrolysed to D-[U- 14 C]apiose and UDP. The percentage of the total radioactivity used that was recovered in the two sugar nucleotides, D-[U- 14 C]apiose and α -D-[U- 14 C]apio-D-furanosyl 1:2-cyclic phosphate ranged from 93 to 100% and averaged 97%.

UDP-[U- 14 C]apiose was readily hydrolysed to

Fig. 4. Hydrolysis of UDP-[U- 14 C]apiose to D-[U- 14 C]apiose and UDP at pH 3.0 and 40°C

The experimental procedure is described in the Materials and Methods section.

D-[U- 14 C]apiose and UDP at pH 3.0 and 40°C (Fig. 4). Kinetic constants are given in Table 5. The UDP-[U- 14 C]xylose present in the sample solutions was not hydrolysed under these conditions.

At least three different preparations of UDP-[U-¹⁴C]apiose were used in each type of experiment described in this section except for the experiment carried out at pH 6.4 and -20°C. UDP-[U-¹⁴C]apiose could be chromatographed on washed paper in solvents (B) and (C) and eluted, all at 4°C, with breakdown being no greater than that obtained when solvent (E) was used.

Discussion

The biosynthesis of D-apiose is considered to occur by conversion of UDP-glucuronic acid into UDP-apiose and CO₂. However, the product, UDP-apiose, has previously only been incompletely characterized and therefore its identity could not be considered established. A complete characterization of UDP-apiose could not be carried out earlier because a procedure for the synthesis and isolation of this sugar nucleotide was lacking. The extreme instability of UDP-apiose made development of such a procedure difficult. However, by using purified UDP-glucuronate cyclase and by following the precautions described a successful procedure was developed. The only radioactive compound contaminating the UDP-[U-¹⁴C]apiose in significant quantities was UDP-[U-¹⁴C]xylose. This contaminant is the result of UDP-glucuronate carboxy-lyase (EC 4.1.1.35) activity present in the UDP-glucuronate cyclase. The procedure has the potential for synthesizing UDP-[U-¹⁴C]apiose in at least 89% yield. To realize this potential the cyclase must be completely separated from the carboxy-lyase or the carboxy-lyase must be prevented from functioning. Since only nanomole quantities of UDP-apiose could be synthesized, the preparation of doubly labelled UDP-apiose was essential for the complete identification of this sugar nucleotide. The identification of UDP-apiose firmly established that the enzyme UDP-glucuronate cyclase catalyses the conversion of UDP-glucuronic acid into UDP-apiose and presumably CO₂.

The results presented showed that UDP-[U-¹⁴C]apiose was extremely labile in the presence of alkali. Information on the alkali-catalysed degradation of sugar nucleotides to sugar cyclic phosphates is limited. UDP-glucose, whose sugar is in the pyranose form, was recovered unchanged after 18h at pH 8.0 and 18°C (Paladini & Leloir, 1952). Under more vigorous conditions it yielded α -D-glucopyranosyl 1:2-cyclic phosphate. In contrast UDP-[U-¹⁴C]apiose, whose sugar is in the furanose form, was much more labile, since 57% of this sugar nucleotide was degraded to α -D-[U-¹⁴C]apio-D-furanosyl 1:2-cyclic phosphate and UMP after 2h at pH 8.0 and 25°C. A possible explanation for the difference in alkali-lability of the two types of sugar nucleotides is given. The five-membered phosphorus-containing ring that is formed on breakdown of sugar nucleotides in alkali

has a conformation that is planar or nearly so. To accommodate this new ring, the C-O bonds on C-1 and C-2 of the furanose and pyranose rings of sugars must be skewed or preferably eclipsed. In furanosides, eclipsing of these two bonds occurs when the furanose ring is in certain envelope conformations. Other conformations that have these bonds slightly skewed would probably also accommodate this new ring. The energy barriers between the various conformations of a furanoside are small or almost non-existent. Therefore the molecules of a furanoside are readily converted into a conformation(s) that can accept the new five-membered ring if they are not already in a reactive conformation(s). On the other hand, for the C-O bonds on C-1 and C-2 of a pyranose ring to be skewed or eclipsed, the pyranose ring must go from the stable 'chair' conformation, partially or completely, into the unstable 'half-chair' conformation. The difference in energy content between the 'chair' conformation of a pyranoside, the normal conformation of almost all pyranosides and one in the 'half-chair' conformation is large. Approximately 42.7 kJ·mol⁻¹ is required to overcome the energy barrier between the 'chair' and 'boat' forms of cyclohexane (Eliel *et al.*, 1965). An obligate (and also the most strained) intermediate of this conversion is the 'half-chair' conformation. Therefore the molecules of a pyranoside have to overcome a relatively large energy barrier to achieve a conformation that can accept the new five-membered ring.

For stereochemical reasons, certain sugar nucleotides cannot yield a sugar cyclic phosphate when degraded by alkali. This is the case when the sugar portion is in the pyranose form and the hydroxyl groups on C-1 and C-2 are in the axial-axial configuration or when the sugar portion is in the furanose form and the hydroxyl groups on C-1 and C-2 are *trans*.

The mechanism of acid hydrolysis of pyranosides is considered to involve the formation of a carbonium-oxonium ion and therefore the 'half-chair' conformation. Assuming that furanosides are also hydrolysed by this mechanism, the same general argument can be used to explain the greater lability of furanoside sugar nucleotides in acid as was used to explain their greater lability in alkali. The mechanism of acid hydrolysis of furanosides has not been established (Capon & Thacker, 1967).

Acid hydrolysis of α -D-[U-¹⁴C]apio-D-furanosyl 1:2-cyclic phosphate should result in the formation of both α -D-[U-¹⁴C]apio-D-furanosyl 1-phosphate and D-[U-¹⁴C]apio-D-furanosyl 2-phosphate. However, in our experiments only the latter compound was observed. α -D-[U-¹⁴C]apio-D-furanosyl 1-phosphate was not observed because it is hydrolysed by acid at a faster rate than is α -D-[U-¹⁴C]apio-D-furanosyl 1:2-cyclic phosphate (Mendicino & Hanna, 1970).

The isolation of UDP-D-galactofuranose from

Penicillium charlesii has been reported (Trejo *et al.*, 1970, 1971). No data were given for the stability of this sugar nucleotide in acid and alkali; however, these workers did state that the sugar nucleotide was very labile in acid. Although UDP-apiose and UDP-D-galactofuranose are the only sugar nucleotides described to date whose sugars are in the furanose form, a number of other sugars are known to occur in biological compounds in the furanose form. In green plants, in addition to D-apiose, L-arabinose (Aspinall, 1959; Hough & Powell, 1960) and D-fructose (Hirst *et al.*, 1950), and in fungi and bacteria, in addition to D-galactose, D-ribose (Berst *et al.*, 1969), L-fucose (Aspinall *et al.*, 1956) and L-streptose (Lemieux & Wolfrom, 1948), have been found in this form. Presumably sugar nucleotides containing these sugars in the furanose form are also present in Nature.

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