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CONVERSION OF UDP-D-GLUCURONIC ACID TO UDP-D-APIOSE AND UDP-D-XYLOSE BY AN ENZYME ISOLATED FROM *LEMNA MINOR*

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SUMMARY

An enzyme catalyzing the synthesis of UDP-D-apiose from UDP-D-glucuronic acid was extensively purified from extracts of *Lemna minor* by a procedure involving adsorption to calcium phosphate, $(NH_4)_2SO_4$ fractionation and chromatography on DEAE-cellulose, hydroxyapatite and Sephadex G-200. The enzyme was purified more than 1000-fold and yields of 20% were consistantly obtained by this procedure. The final preparation was nearly homogeneous as determined by polyacrylamide-gel electrophoresis and sucrose-density centrifugation. The molecular weight of the enzyme measured by chromatography on Sephadex G-200 and sucrose-density centrifugation was 110 000 ± 10 000. Some of the kinetic properties of the enzyme were examined. Dithiothreitol or cysteine and NAD were required for maximal activity, UDP, NH_4^+ and NADH inhibited the reaction. The enzyme forms UDP-D-xylose as well as UDP-D-apiose. The K_m of the enzyme for UDP-D-glucuronic acid was 2 μM.

The mechanism of the reaction was examined with specifically labeled substrates. The results of experiments with UDP-D-[3-^{14}C, 3-^{3}H]glucuronic acid and UDP-D-[3-^{14}C, 4-^{3}H]glucuronic acid showed that essentially all of the ^{3}H from C-4 of UDP-D-glucuronic acid was transferred to the free hydroxymethyl group of D-apiose, whereas only about 40% of the ^{3}H from C-3 of UDP-D-glucuronic acid was retained in the hydroxymethyl group of D-apiose. These studies further showed that C-3 of UDP-D-glucuronic acid was converted to C-4′ of D-apiose. The results indicate that a hydride transfer and isomerization reaction may be involved in the conversion of UDP-D-glucuronic acid to UDP-D-apiose.

INTRODUCTION

The branched-chain sugar, D-apiose (2-C-hydroxymethyl-D-ribose), is found in *Lemna minor* and many seaweeds as a constituent of pectins and other cell wall polysaccharides [1–5]. In parsley it is present as a component of the flavone, apiin [1]. Previous isotopic tracer studies with various labeled substrates have shown that D-glucose can serve as the sole precursor for all of the carbon atoms of D-apiose [6–9]. Further evidence for the mechanism of biosynthesis of D-apiose was obtained by Roberts et al. [10] who showed that *myo*-[2-^{14}C]inositol was incorporated into the D-apiosyl and D-xylosyl moieties of cell-wall polysaccharides of *Lemna gibba*. *Myo-*

inositol is an effective precursor of D-glucuronic acid in higher plants, and it is specifically utilized in the biosynthesis of uronic acid and pentose residues of cell wall polysaccharides [11, 12]. These and other results provided further evidence that D-apiose was derived from UDP-D-glururonic acid [13].

Small amounts of UDP-D-apiose (0.18 μmole) were reported to be present in a UDP-sugar fraction (120 μmoles) isolated from an ethanol extract of parsley plants [14]. Gustine and Kindel [15] using cell-free extracts of *Lemna minor* showed that labeled UDP-D-glucuronic acid was converted to small amounts of UDP-D-apiose and a ^{14}C-labeled phosphate ester of D-apiose. A number of studies have shown that UDP-D-glucuronic acid, uniformly labeled with ^{14}C in the glucuronic acid moiety, is converted to UDP-D-apiose and apiose phosphate derivatives upon incubation with NAD and extracts prepared from parsley and *Lemna minor* [16, 17]. Enzymes which catalyze the conversion of UDP-D-glucuronic acid to UDP-D-apiose and UDP-D-xylose have been partially purified from parsley and *Lemna minor* [18–22]. Kelleher et al. [23] using specifically labeled UDP-D-glucuronic acid showed that a hydride shift occurs from C-4 of UDP-D-glucuronic acid to C-4′ of D-apiose during the formation of UDP-D-apiose from UDP-D-glucuronic acid. This transfer is similar to that observed by Schutzbach et al. [24] during the formation of UDP-D-xylose from UDP-D-glucuronic acid by a purified UDP-D-glucuronic acid carboxy-lyase isolated from wheat germ.

The present communication describes the isolation of UDP-D-apiose synthetase from *Lemna minor* and reports some of the physical and chemical properties of the purified enzyme. The nature of the intermediate reactions involved in the conversion of UDP-D-glucuronic acid to UDP-D-apiose was examined with specifically labeled substrates.

MATERIALS AND EXPERIMENTAL PROCEDURES

Lemna minor were cultured in the laboratory under conditions of continuous light in large tanks containing only minimal salts [25, 26]. Snails were used to control the growth of algae, and large quantities of plants, 1000 g wet weight, which completely covered the surface of the water in the tanks, were harvested every 2 weeks. In growth experiments about 100 sterilized fronds were placed in flasks containing sterilized salt solution, and they were grown at room temperature in continuous light. Germination of parsley seeds was carried out under sterile conditions in 0.5% agar in petri dishes. The seeds, obtained from Capitol Seeds Co., Columbus, Ohio, were rinsed in 95% ethanol and they were then soaked for 15 min in 2.5% (w/w) NaOCl and rinsed with sterile water. They were kept at room temperature for 48 days.

Enzyme assays

The rate of conversion of UDP-D-[U-^{14}C]glucuronic acid to UDP-D-[^{14}C]-apiose and UDP-D-[^{14}C]xylose was measured by quantitative paper chromatography. The standard reaction mixture was incubated at 30 °C for 15 min and contained in a total volume of 0.08 ml, 1.7 μM UDP-D-[U-^{14}C]glucuronic acid ($3.9 \cdot 10^8$ cpm/μmole), 25 mM Tris–HCl (pH 7.5), 1 mM dithiothreitol, 2 mM NAD, 0.1 M KCl, 2 mM $MgCl_2$, 1 mM *P*-enolpyruvate, 0.1 unit of pyruvate kinase, and an appropriate amount of enzyme. The reaction was stopped by the addition of 0.05 ml of glacial acetic acid,

0.2 μmole each of unlabeled D-apiose and D-xylose was added, and the solution was heated at 100 °C for 10 min to hydrolyze the labile phosphate derivatives of these sugars. The solution was streaked on washed Whatman 3 MM paper and the chromatogram was developed with reference standards in two solvent systems. The paper was developed with pyridine–ethyl acetate–water (2:8:1, by vol.) for 5 h and it was dried, and irrigation was repeated with butanol–ethanol–water (52:32:16, by vol.) for 5 h. The areas containing D-apiose and D-xylose were detected by using the reference standards and the corresponding radioactive strips were cut out, transferred to scintillation vials and counted. Standard curves were prepared by adding ^{14}C-labeled D-apiose and D-xylose to a complete reaction mixture at zero time. One unit of activity in this assay is defined as the amount of enzyme required to convert 1 μmole of UDP-D-glucuronic acid to 1 μmole of UDP-D-apiose or UDP-D-xylose per min. Specific activity is expressed as units/mg of protein.

Preparation of α-D-apiofuranosyl 1,2-cyclic phosphates

The α-D-furanosyl 1,2-cyclic-*P* and α-D-apio-L-furanosyl 1,2-cyclic-*P* diesters of D-apiose were prepared by treating the β-isomers of D-apiose tetraacetate with anhydrous crystalline phosphoric acid [17]. The two corresponding monophosphate isomers, which were also formed, were converted to the 1,2-cyclic phosphodiester derivatives by an acid catalyzed migration reaction of the phosphate group at pH 1 and 26 °C. The cyclic phosphate sugars were isolated by chromatography on Dowex 1-HCO_3^- columns and by paper chromatography as described previously [17]. The pooled fractions from Dowex 1-HCO_3^- columns were concentrated under reduced pressure and samples were applied to sheets of Whatman 3 MM paper. The paper was washed with *n*-butanol–ethanol–water (52:32:16, by vol.) and it was then washed with *n*-propanol–ethyl acetate–water (7:1:2, by vol.) to remove LiCl and other salts. The phosphate esters which remained near the origin of the chromatogram were eluted from the paper with water and the solutions were concentrated by evaporation. The preparations were further purified by paper chromatography in several solvent systems [17]. Kindel and Watson [27] have also prepared α-D-apiofuranosyl 1,2-cyclic-*P* from UDP-D-apiose.

UDP-D-apiose, formed in reaction mixtures from UDP-D-glucuronic acid, was converted to α-D-apio-D-furanosyl 1,2-cyclic phosphate under alkaline conditions without passing through the free aldehydo form [17]. Periodate oxidation of the cyclic phosphate derivative proceeds with a rapid consumption of only one equivalent of periodate at 26 °C and pH 6.5 and the free hydroxymethyl group of the D-apiose cyclic-*P* derivative is converted to formaldehyde. Formaldehyde, derived from the C-4′ group of D-apiose 1,2-cyclic-*P*, was released at the same rate as periodate was utilized, and after 3 h 0.98 mole of formaldehyde per mole of sugar phosphate was formed. The C-4 and C-4′ groups of D-apiose formed from UDP-D-glucuronic acid can be separated by this procedure.

Degradation of D-apiose 1,2-cyclic-P derived from specifically labeled UDP-D-glucuronic acid by periodate oxidation

The labeled cyclic phosphates were isolated from reaction mixtures by paper chromatography in several solvent systems including isopropanol–ammonia–water (7:1:2, by vol.) and methyl cellosolve–acetic acid–pyridine–water (8:1:4:1, by vol.).

Those areas which chromatographed with unlabeled D-apiose 1,2-cyclic-*P* were eluted and counted. The $^{3}H/^{14}C$ ratio was determined in the substrate and in the product during the course of the reaction.

To remove the hydroxymethyl group at the C-4′ position of the D-apiose moiety, the D-apiose 1,2-cyclic-*P* was treated with $NaIO_4$ under mild conditions [17] and the formaldehyde derived from C-4′ was isolated as the crystalline dimedon derivative and counted. The other oxidized product, 1,2-cyclic phosphate-3-ketotetrose, was reduced by treatment with $NaBH_4$. The phosphate group was removed by heating with 0.1 M HCl at 100 °C for 10 min and the resulting tetrose was isolated by paper chromatography and counted.

*Preparation of UDP-*D*-[3-^{3}H]glucuronic acid, UDP-*D*-[4-^{3}H]glucuronic acid and specifically labeled UDP-*D*-[^{14}C]glucuronic acid*

Specifically labeled D-[3-^{3}H]glucose, D-[4-^{3}H]glucose, D-[U-^{14}C]glucose, D-[1-^{14}C]glucose and D-[3-^{14}C]glucose were converted to the corresponding UDP-D-glucose derivatives by incubation with a coupled enzyme system as described in previous studies [28]. The reaction mixture, in each case, was incubated at room temperature for 3 h and contained in a total volume of 0.2 ml; 10 mM Tris–HCl, pH 7.5, 10 mM UTP, 10 mM $MgCl_2$, 50 mM KCl, 3 mM *P*-enolpyruvate, specifically labeled D-glucose, 1 nmole of D-glucose 1,6-P_2, 2 units of hexokinase, 2 units of phosphoglucomutase, 1.5 units of UDP-glucose pyrophosphorylase, 1 unit of inorganic pyrophoglucomutase, 1 unit of inorganic pyrophosphatase and 1 unit of pyruvate kinase. UDP-D-glucose was isolated by ionexchange and paper chromatography with several solvent systems. The corresponding UDP-D-glucuronic acid derivatives were prepared by treating labeled UDP-glucose with UDP-D-glucose dehydrogenase [24].

Materials and methods

D-[1-^{14}C]Glucose and D-[3-^{14}C]glucose were purchased from New England Nuclear. D-[3-^{3}H]Glucose (2.5 Ci/mmole), D-[4-^{3}H]glucose (7.1 Ci/mmole) and UDP-D-[U-^{14}C]glucuronic acid (0.3 Ci/mmole) were obtained from Amersham Searle. Hexokinase, phosphoglucomutase, UDP-glucose dehydrogenase, were purchased from Sigma. UDP-glucose pyrophosphorylase was prepared from rabbit muscle by the method of Villar-Palasi and Larner [29]. Enzyme grade Tris (General Biochemicals) and $(NH_4)_2SO_4$ (Mann) were used.

Analysis of sugars by paper chromatography on Whatman 3 MM paper previously washed with 1% oxalic acid was carried out with the following solvent systems; *n*-butanol–pyridine–water (6:4:3, by vol.), pyridineethyl acetate-water (2:8:1, by vol.) [30], butanol–ethanol–water (52:32:16, by vol.) [31], ethyl acetate–acetic acid–water (3:3:1, by vol.) [32]. Sugars were detected with the $AgNO_3$ and alcoholic NaOH reagent [33]. The following solvents were used to separate sugar phosphates and nucleoside diphosphate sugars by paper chromatography, isopropanol–ammonia (spec. gravity 0.9)–water (7:1:2, by vol.) [34], methyl cellosolve–acetic acid–pyridine–water (8:1:4:1, by vol.) [35], 95% ethyl alcohol–1 M ammonium acetate, pH 7.3 (7:3, v/v) [36], 95% ethyl alcohol–1 M ammonium acetate, pH 3.8 (7:3, v/v) [36], isobutyric acid–1 M NH_4OH–0.1 M EDTA (50:30:1, by vol.), ethyleneglycol dimethyl ether–methylethyl ketone–0.5 M morpholinium tetraborate, pH 8.6, and 0.01 M EDTA (7:2:3, by vol.) [37]. Nucleoside diphosphate sugars were detected

with ultraviolet light and sugar phosphates were located with the sulfosalicylic acid reagent [35].

The concentration of D-apiose and D-apiose phosphates was determined by a modification of the fructose–H_2SO_4 method for tetroses [38]. The utilization of periodate was measured spectrophotometrically by following the decrease in light absorption at 222.5 nm [39]. Formaldehyde was assayed by the chromotropic acid method [40]. Reduction with borohydride was carried out according to the method of Abdel-Akher et al. [41]. Protein was measured by the method of Lowry et al. [42]. Sucrose-density centrifugation was performed according to Martin and Ames [43] and molecular weight determinations on Sephadex G-200 columns were carried out by the method of Andrews [44]. Radioactivity measurements were carried out in a Packard scintillation counter with Bray's solution or the solvent system of Patterson and Greene [45]. Discriminator settings were selected for maximum counting efficiency of ^{14}C and ^{3}H with only about 30% spillover of ^{14}C into the ^{3}H channel under these conditions.

RESULTS

Rate of synthesis and content of D-*apiose in Lemna minor and parsley*

Germinating parsley seeds and growing *Lemna minor* can incorporate $^{14}CO_2$, D-glucose and D-glucuronic acid into the D-apiosyl moiety of the flavinol, apiin and apiogalacturonans, respectively. The effects of germination on the content and rate of synthesis of D-apiose in young parsley plants was compared with the activity of the same enzyme in growing *Lemna minor* under sterile conditions for 35 days. The results shown in Fig. 1 illustrate the differences in the rate of synthesis of D-apiose in these tissues. The enzyme cannot be assayed in crude extracts of these tissues because of interferring side reactions which hydrolyze the substrate. A potent heat-labile inhibitor of the reaction is also present in crude enzyme preparations. However, it can be assayed after precipitation with $(NH_4)_2SO_4$ to 70% saturation and chro-

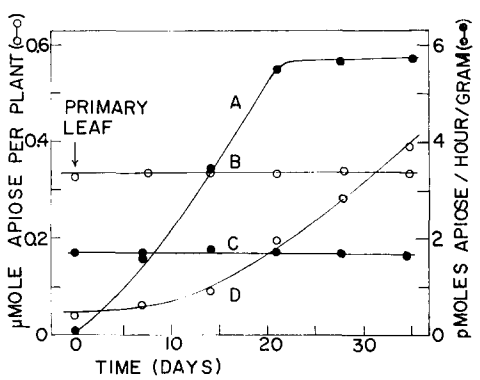

Fig. 1. Rate of synthesis and content of D-apiose in *Lemna minor* and parsley. Curve A, apiose synthetase activity in parsley plants following the appearance of the primary leaf. Curve D, the amount of D-apiose present in parsley plants. Curve C, apiose synthetase activity in *Lemna minor*. Curve B, the amount of D-apiose present in samples of *Lemna minor* harvested at the same time. The assays were carried out as described in the text.

matography on a small Sephadex G-200 column (1.5 cm × 30 cm). All of the enzyme activity in the extract is recovered by this procedure. The addition of samples of the purified enzyme to the crude extract resulted in a quantitative recovery of activity, which was greater than a control sample by the amount of the purified enzyme which was added. The content of D-apiose was measured on samples after hydrolysis with dilute acid as described previously [6].

The amount of D-apiose in parsley plants increased for about 20 days after the appearance of the primary leaf. The amount of D-apiose synthetase also increased during this period as shown in Fig. 1. The quantity of D-apiose isolated from this tissue was usually equal to the amount of apiin present, which indicated that nearly all of the D-apiose in parsley occurs as the flavone glycoside [6]. The results of previous studies clearly indicated that leaves and seeds in parsley contain the highest concentration of apiin [6]. It is possible that the distribution of apiin in different tissues of the parsley plant other than the site of synthesis may be influenced by time and the age of the plant.

In marked contrast, the content of D-apiose and amount of D-apiose synthetase in growing *Lemna minor* remained constant throughout the same period of time. This effect may be due to the fact that D-apiose in this plant is present in cell wall polysaccharides and the content and synthesis of these materials may remain relatively constant under these conditions. The studies described in this report were performed with the enzyme isolated from *Lemna minor*.

Isolation of UDP-D-apiose synthetase from Lemna minor

Unless otherwise indicated all operations were carried out at 3 °C and centrifugations were performed at 27 000 × *g* for 10 min. The plants were washed with distilled water and 1300 g (wet wt) were suspended in 3 l of 0.1 M Tris–HCl, pH 8.0–0.2 M KCl–1% polyvinylpyrrolidone-10 (Sigma)–0.02 M 2-mercaptoethanol–0.001 M EDTA. The cells were disrupted by treatment with a Polytron homogenizer, Brinkmann Inst., Westbury, N.Y., for 10 min. The preparation was treated for 1-min intervals with intermittant cooling. The resulting suspension was filtered through cheesecloth and the extract was centrifuged to remove additional insoluble material (Fraction 1, Table I). The activity of the enzyme is inhibited in this preparation. An aliquot of the crude extract was precipitated with $(NH_4)_2SO_4$ and chromatographed on a Sephadex G-200 column as described previously. A quantitative recovery of the enzyme activity can be obtained by this procedure and the activity at this stage was determined on this sample.

The enzyme was precipitated from the supernatant solution by the addition of solid $(NH_4)_2SO_4$ to 70% saturation at 3 °C and the precipitate was suspended in 30 ml of 0.02 M Tris–HCl, pH 8.0–1 mM dithiothreitol. Insoluble material was removed by centrifugation and it was re-extracted with another 30 ml of buffer. The extracts were combined and salt was removed by chromatography on Sephadex G-25 columns (2.2 cm × 30 cm) with a solution containing 0.05 M Tris–HCl, pH 8.0–1 mM dithiothreitol. The solution was stirred at 3 °C and 60 ml of a slurry containing calcium phosphate gel, 50 mg (dry wt) per ml, was slowly added. The gel was collected by centrifugation, and it was washed twice by resuspending it in 200 ml of ice-cold 0.05 M Tris–HCl, pH 8.0–1 mM dithiothreitol and centrifuging the resulting slurry. The enzyme was eluted from the washed gel by two successive extractions with 150 ml

TABLE I

ISOLATION OF UDP-D-APIOSE SYNTHETASE FROM *LEMNA MINOR*

The enzyme was assayed as described in the text.

Fraction	Volume (ml)	Protein (mg/ml)	Spec. act. (units × 10^6/mg)		Yield (%)
			Apiose	Xylose	
1 Crude extract	3050	2.0	0.28	0.21	100
2 Elution from calcium phosphate gel	300	1.5	2.9	2.2	76
3 Chromatography on DEAE-cellulose	260	0.18	26.1	20.7	71
4 Chromatography on hydroxyapatite	5	0.9	165	122	44
5 Chromatography on Sephadex G-200	2	0.5	340	270	20

of 0.02 M Tris–HCl, pH 8.0–0.02 M potassium citrate–1 mM dithiothreitol. The gel was removed by centrifugation after each extraction and the supernatant solutions were combined (Fraction 2).

The solution was diluted with an equal volume of buffer and it was passed into a DEAE-cellulose column (5.8 cm × 10 cm), which was previously equilibrated with 0.02 M Tris–HCl, pH 8.0–1 mM dithiothreitol. The column was washed with 100 ml of 0.05 M Tris–HCl, pH 8.0, and then with 250 ml of 0.05 M Tris–HCl, pH 8.0–0.05 M KCl–1 mM dithiothreitol. The enzyme was eluted with about 200 ml of 0.05 M Tris–HCl, pH 8.0–0.2 M KCl–1 mM dithiothreitol. The activity appeared in the first protein peak after about 20 ml of solution had passed into the column (Fraction 3).

The activity fractions from the previous step were combined and diluted with one volume of distilled water. The preparation was then applied to a hydroxyapatite column (4.4 cm × 8 cm) which was previously equilibrated with 0.05 M Tris–HCl, pH 7.5–1 mM dithiothreitol. Afterwards the column was washed with 30 ml of the same buffer. The enzyme was eluted with a linear gradient formed with 200 ml of 0.005 M potassium phosphate, pH 7.5–1 mM dithiothreitol in the mixing chamber and 200 ml of 0.1 M potassium phosphate, pH 7.5–1 mM dithiothreitol in the reservoir. The D-apiose synthetase activity was found in a single protein peak which emerged from the column after about 50 ml of the eluting solution had been collected. The fractions containing significant activity were combined and the solution was concentrated by use of a Diaflo ultrafiltration cell (Amicon Corp., Lexington, Mass.). Salt was removed by chromatography on a Sephadex G-25 column as described previously.

The enzyme in 5 ml was passed into a Sephadex G-200 column (2.2 cm × 30 cm) which was previously equilibrated against 0.05 M Tris–HCl, pH 7.5–1 mM dithiothreitol. The column was developed with this same buffer. The UDP-D-apiose synthetase activity was eluted in a single nearly symmetrical protein peak and all of the fractions (5 ml) had about the same specific activity. The fractions with the highest activity were combined and concentrated as described previously (Fraction 5). A purification of about 1000-fold over the crude extract with a yield of about 20 % was obtained by this procedure.

Properties of the purified enzyme

The enzyme was stable for about a month when it was stored frozen at −20 °C in 0.05 M Tris–HCl, pH 7.5–1 mM dithiothreitol. The enzyme had a broad pH optimum between pH 7.0 and 8.5 in the standard assay system.

As shown in Fig. 2, the activity of the purified enzyme was linear with time and proportional to enzyme concentration in the standard assay system. During puri-

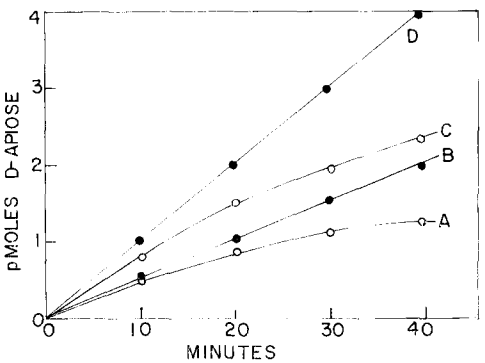

Fig. 2. Proportional relation of enzyme activity with respect to time and protein concentration in the presence and absence of pyruvate kinase. The standard assay was used except that pyruvate kinase was omitted in Curve A and Curve C. The reaction mixtures in Curves A and B contained 2 μg of enzyme (Fraction 3) and those in Curves C and D contained 4 μg of enzyme.

fication of the enzyme it was necessary to add pyruvate kinase to the assay mixture. The influence of pyruvic kinase on the velocity of the reaction with a partially purified preparation is shown in Fig. 2. In the absence of pyruvate kinase an inhibition of the reaction was observed after about 10 min. When pyruvate kinase was present, Curves B and D, the reaction was linear for about 40 min. This inhibition was presumably due to the accumulation of UDP formed by hydrolysis of the nucleoside diphosphate derivatives. When UDP, 30 μM, was added to the reaction mixture initially the rate of formation of UDP-D-apiose and UDP-D-xylose decreased. At a concentration of 1 mM UMP and UTP had no effect on the activity of the enzyme. In other experiments the dependence of the initial velocity upon the concentration of UDP-D-glucuronic acid was measured at different concentrations of UDP. The inhibition by UDP was found to be competitive with respect to substrate, which suggested that UDP-D-glucuronic acid and UDP might be bound to the same site on the enzyme. The apparent K_i for UDP calculated from this data was 5 μM. Since UDP was a potent inhibitor of the reaction all of the samples of UDP-D-glucuronic acid used in these studies were treated with pyruvate kinase to convert any UDP which might be present to UTP. The reaction was also strongly inhibited by NH_4^+ and high concentrations of salt. Excess salt and particularly $(NH_4)_2SO_4$ was removed from all enzymes and reagents before they were used in the assay mixtures.

Determination of molecular weight by sucrose-density centrifugation and chromatography on Sephadex G-200

Polyacrylamide-gel electrophoresis of the purified enzyme at pH 8.9 and 4.3

according to the procedure of Davis [46] showed the presence of a single major component. About 50 μg of the enzyme was applied to the gel at each pH. Two minor diffuse bands were occasionally observed at pH 8.9 with some preparations. The elution pattern of the purified preparation on Sephadex G-200 also indicated that the enzyme was nearly homogeneous. The enzyme was eluted in a single symmetrical protein peak and all of the fractions taken across the peak had a constant specific activity.

The molecular weight of D-apiose synthetase was determined by gel filtration on a Sephadex G-200 column (2.2 cm $\times$ 27 cm). The elution volume of the purified enzyme was compared with the elution volumes of aldolase, lactate dehydrogenase, hexokinase and alcohol dehydrogenase on the same column. The elution volume of D-apiose synthetase was 70 ml. Calculations based on plots of the log of the molecular weights against elution volumes of the standards according to the method of Andrews [44] yielded a molecular weight of 110 000 $\pm$ 7000 for D-apiose synthetase. The molecular weight of the enzyme was also estimated by sucrose-density centrifugation. Samples were spun in a SW-39 rotor for 8 h at 37 000 rev./min. The relative distribution of lactate dehydrogenase, alcohol dehydrogenase and catalase were compared with that of D-apiose synthetase after centrifugation in a 5–20% sucrose gradient. The molecular weight of the enzyme calculated according to the method of Martin and Ames [43] was 110 000 $\pm$ 5000. The values obtained by this procedure were in good agreement with those obtained by gel filtration.

Influence of the concentration of UDP-D-glucuronic acid on the velocity of the reaction

The purified enzyme catalyzes the synthesis of both UDP-D-apiose and UDP-D-xylose, presumably from intermediates of the same reaction sequence. The effect of the concentration of UDP-D-glucuronic acid on the rate of formation of UDP-D-apiose and UDP-D-xylose was examined and the results are summarized in Fig. 3. The apparent Michaelis constants calculated from data obtained in several experiments was 2 μM for the formation of both UDP-D-apiose and UDP-D-xylose from UDP-D-glucuronic acid. These results further indicate that both of these nucleoside diphosphate sugars are formed from UDP-D-glucuronic acid by the action of the

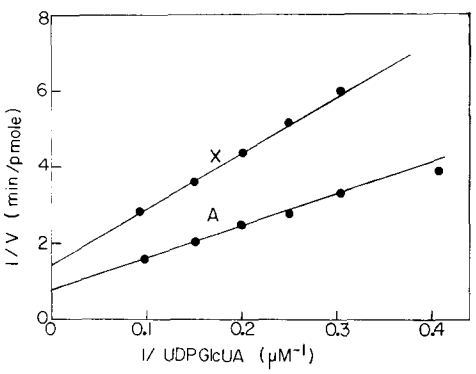

Fig. 3. The dependence of the initial velocity on the concentration of UDP-D-glucuronic acid (UDP-GlcUA) as a function of the rate of formation of UDP-D-apiose (Curve A) and UDP-D-xylose (Curve X). Varying amounts of UDP-D-glucuronic acid were added to the standard reaction mixture and the amount of D-apiose and D-xylose formed was assayed as described in Experimental Procedures.

same enzyme and that the same rate limiting step is involved in the formation of both compounds. The ratio of UDP-D-apiose/UDP-D-xylose formed by all of the fractions obtained during the purification of the enzyme was 1.2 to 1.4, as seen in Table I. This observation is consistant with the results obtained in kinetic experiments which suggest that the same enzyme is responsible for the formation of both products.

The enzyme requires the addition of NAD^+ for maximal activity as reported earlier [17], and NADH inhibits the reaction competitively with respect to NAD^+. Only 5% of the maximal activity was observed with the purified enzyme in the absence of added NAD^+. The apparent Michaelis constant of the enzyme for NAD^+ was 30 μM in the standard assay system.

Transfer of tritium from UDP-D-[4-^{3}H]glucuronic acid, UDP-D-[3-^{3}H]glucuronic acid and ^3H_2O to UDP-D-apiose

Specifically labeled substrates were converted to UDP-D-apiose and the position of the label was determined by periodate oxidation of the D-apiose 1,2-cyclic-*P* formed from the nucleotide sugar in alkali. This procedure permitted the isolation and identification of ^{3}H and ^{14}C present in the C-4′ group of D-apiose. The results obtained in several experiments with UDP-D-[4-^{3}H]glucuronic acid and UDP-D-[3-^{3}H]glucuronic acid are summarized in Table II. The specific activity of the D-

TABLE II

SPECIFIC ACTIVITIES OF α-D-APIO-D-FURANOSYL 1,2-CYCLIC-*P*, FORMALDEHYDE AND TETROSE PHOSPHATE OBTAINED WITH UDP-D-[4-^{3}H]GLUCURONIC ACID AND UDP-D-[3-^{3}H]GLUCURONIC ACID

The substrates were prepared as described in the text. The reaction mixtures in each case were incubated at 30 °C for 1 h and contained in a total volume 0.2 ml: 0.05 mM Tris–HCl, pH 7.5, 2 mM NAD^+, 2 mM dithiothreitol, 25 μg of purified enzyme and 10 nmoles of labeled UDP-D-glucuronic acid. Afterwards 5 μmoles of carrier D-apiose 1,2-cyclic-*P* was added and the pH of the reaction mixture was adjusted to 9.0 by the addition of KOH. The solution was kept at room temperature for 30 h and the labeled D-apiose 1,2-cyclic-*P* was then reisolated and converted to formaldehyde and tetrose phosphate as described in Experimental Procedures.

Experiment	Specific activity (cpm/μmole)
UDP-D-[4-^{3}H]glucuronic acid	$1.14 \cdot 10^6$
D-Apiose 1,2-cyclic-*P*	590
Formaldehyde	560
Tetrose phosphate	20
UDP-D-[3-^{3}H]glucuronic acid	$1.15 \cdot 10^6$
D-Apiose 1,2-cyclic-*P*	320
Formaldehyde	310
Tetrose phosphate	10

apiose 1,2-cyclic-*P* derived from UDP-[4-^{3}H]glucuronic acid, 590 cpm/μmole, was almost twice as great as that obtained from UDP-D-[3-^{3}H]glucuronic acid, 320 cpm/μmole. The specific activities of the UDP-D-glucuronic acid samples were nearly equal. All of the ^{3}H originally present in the UDP-D-glucuronic acid was found in the formaldehyde derived from C-4′ of the D-apiose 1,2-cyclic-*P* in both cases, and

the other 4 carbons in the tetrose phosphate contained less than 5% of the original radioactivity. These results indicated that almost all of the hydrogen at C-4 of UDP-D-glucuronic acid was transferred to C-4′ of D-apiose and that about 50% of the hydrogen at C-3 was also transferred to the same carbon during this conversion.

Transfer of ^{14}C and ^{3}H from specifically labeled UDP-D-glucuronic acid to UDP-D-apiose

Further evidence for the mechanism of the reaction catalyzed by the purified enzyme was obtained with doubly labeled substrates. The UDP-D-[3-^{14}C, 3-^{3}H]glucuronic acid, UDP-D-[3-^{14}C4-^{3}H]glucuronic acid and UDP-D-[1-^{14}C, 3-^{3}H]glucuronic acid used in these experiments were prepared by mixing UDP-D-[3-^{14}C]glucuronic acid, UDP-D-[1-^{14}C]glucuronic acid, UDP-D-[3-^{3}H]glucuronic acid and UDP-D-[4-^{3}H]-glucuronic acid so that the $^{3}H/^{14}C$ ratio in each of the doubly labeled substrates was approximately 1. The results presented in Table III clearly establish that C-3 of UDP-

TABLE III

SPECIFIC ACTIVITIES OF α-D-APIO-D-FURANOSYL 1,2-CYCLIC-P, FORMALDEHYDE AND TETROSE PHOSPHATE OBTAINED FROM UDP-D-[3-^{14}C, 3-^{3}H]GLUCURONIC ACID, UDP-D-[3-^{14}C, 4-^{3}H]GLUCURONIC ACID AND UDP-D-[1-^{14}C, 3-^{3}H]GLUCURONIC ACID

The conditions and procedures were the same as those described in Table II except for the substrates. GlcUA, glucuronic acid.

Experiment	Spec. act. (cpm/μmole)		$^{3}H/^{14}C$
	^{14}C	^{3}H	
UDP-D-[3-^{14}C, 3-^{3}H]GlcUA	$1.20 \cdot 10^6$	$1.15 \cdot 10^6$	0.97
D-Apiose 1,2-cyclic-*P*	780	370	0.48
Formaldehyde	760	360	0.47
Tetrose phosphate	40	10	—
UDP-D-[3-^{14}C, 4-^{3}H]GlcUA	$1.20 \cdot 10^6$	$1.14 \cdot 10^6$	0.95
D-Apiose 1,2-cyclic-*P*	630	610	0.98
Formaldehyde	590	580	0.97
Tetrose phosphate	30	10	—
UDP-D-[1-^{14}C, 3-^{3}H]GlcUA	$1.10 \cdot 10^6$	$1.15 \cdot 10^6$	1.05
D-Apiose 1,2-cyclic-*P*	710	330	0.46
Formaldehyde	40	290	—
Tetrose phosphate	680	20	—

D-glucuronic acid is converted to the C-4′ group of UDP-D-apiose. Thus, the specific activity of ^{14}C in D-apiose 1,2-cyclic-*P* and formaldehyde derived from UDP-D-[3-^{14}C, 3-^{3}H]glucuronic acid and UDP-D-[3-^{14}C, 4-^{3}H]glucuronic acid are nearly the same, whereas the ^{14}C in the D-apiose, 1,2-cyclic-*P* derived from UDP-[1-^{14}C, 3-^{3}H]-glucuronic acid is found principally in the tetrose phosphate. The ratio of $^{3}H/^{14}C$ in the D-apiose 1,2-cyclic-*P* and formaldehyde formed from UDP-D-[3-^{14}C, 4-^{3}H]glucuronic acid indicate that nearly all of the ^{14}C at 3-C and the ^{3}H at C-4 of the glucuronic acid moiety has been transferred to C-4′ of D-apiose during the reaction. Only about 40–50% of the ^{3}H at C-3 in UDP-D-[3-^{14}C, 3-^{3}H]glucuronic acid and UDP-[1-^{14}C, 3-^{3}H]glucuronic acid is transferred to C-4′ of UDP-D-apiose.

These results indicate that the free hydroxymethyl group of D-apiose is formed from carbon atom 3 and protons originally present at C-3 and C-4 of UDP-glucuronic acid.

DISCUSSION

The purified enzyme described in this report is probably responsible for the biosynthesis of both UDP-D-apiose and UDP-D-xylose in *Lemna minor*. These compounds in turn act as glycosyl donors in the transfer of these sugars to cell wall polysaccharides in *Lemna minor*. The molecular weight and kinetic properties of this enzyme are quite similar to the enzyme isolated from parsley [22]. However, in this tissue one of the products, UDP-D-apiose, is utilized almost exclusively in the synthesis of flavone glycoside, apiin, while the other UDP-D-xylose is presumably used in the synthesis of polysaccharides. The results of the present study indicate that the synthesis and regulation of this enzyme in these tissues may be different. The activity and amount of enzyme in parsley varies with the age of the plant, whereas in *Lemna minor* the amount and activity of the enzyme remains relatively constant.

The properties of the enzyme and the results obtained with specifically labeled UDP-D-glucuronic acid as the substrate are consistant with the reaction sequence shown in Fig. 4 [7]. The evidence obtained thus far suggests that both UDP-D-apiose and UDP-D-xylose are formed from a common intermediate, and that UDP-D-xylose may be formed before UDP-D-apiose by reduction of the 4-keto-pyranosylpentose shown in Fig. 4. Experiments with labeled UDP-D-[3-^{14}C, 3-^{3}H]glucuronic acid or

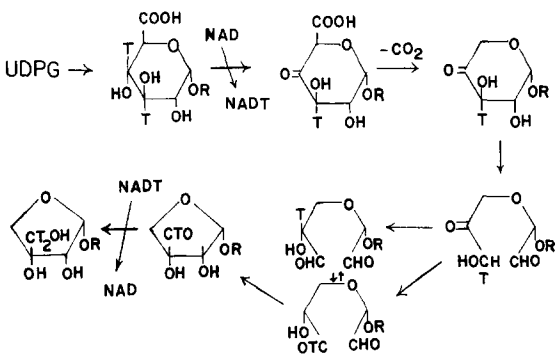

Fig. 4. Proposed reaction sequence for the transfer of ^{3}H from specifically labeled UDP-D-glucuronic acid to UDP-D-apiose.

the same compound labeled at C-4 with ^{3}H showed that essentially all of the tritium from the C-4 of UDP-D-glucuronic acid moiety was transferred to the free hydroxymethyl group of D-apiose, whereas only about 50% of the tritium from the C-3 group of UDP-D-glucuronic acid was retained in the hydroxymethyl group of D-apiose. Our studies further show that C-3 of UDP-D-glucuronic acid is converted to C-4′ of D-apiose. These results are in agreement with those obtained by Kelleher et al. [23, 47] and Baron et al. [48] with an enzyme isolated from parsley.

The tritium at C-4 of UDP-D-glucuronic acid may be transferred to NAD^{+} to

form a 4-keto intermediate as shown in the figure. The hydride shift from C-4 of UDP-D-glucuronic acid to the C-4′ of D-apiose catalyzed by enzyme bound NAD without exchange with the protons of water is characteristic of many of the enzymes catalyzing isomerization and oxidation–reduction reactions between nucleoside diphosphate sugars [49]. Cleavage between C-2 and C-3, isomerization and intramolecular aldol condensation may result in the formation of an intermediate in which C-3 of glucuronic acid is converted to the branched chain carbon atom of D-apiose. The enzyme bound NAD^{3}H then reduces the aldehyde group at C-4′ to the hydroxymethyl group of D-apiose. This mechanism would explain the hydride shift from C-4 of the glucuronic acid moiety to the hydroxymethyl group of D-apiose. These results also explain the requirement for catalytic amounts of NAD^+ in the formation of D-apiose. The loss of ^{3}H from C-3 of the glucuronic acid moiety could occur in the isomerization step. About half of the ^{3}H would be lost at this stage. These results are consistant with the appearance of ^{3}H from the C-3 and C-4 groups of UDP-glucuronic acid in the hydroxymethyl group of D-apiose. The tritium at C-3 of the dialdol intermediate might be expected to exchange with water in a stereospecific isomerase reaction [50]. This exchange could explain the loss of 50% of the ^{3}H from C-3 of UDP-D-[3-^{3}H]-glucuronic acid and the incorporation of ^{3}H from ^3H_2O into UDP-D-apiose.

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