Eur. J. Biochem. *38*, 153–159 (1973)

Further Studies on the Mechanism of Action of UDP-Apiose/UDP-Xylose Synthase from Cell Cultures of Parsley

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(Received May 14/June 26, 1973)

1. When uridine diphospho-D-[U-^{14}C]xylose was incubated with the UDP-apiose/UDP-xylose synthase from cell suspension cultures of parsley no radioactivity was detected in UDP-apiose (UDP-Api).

2. UDP-D-glucuronic acid (UDP-GlcUA) labeled with tritium at either C-4 or C-5 was converted to UDP-Api with UDP-Api/UDP-Xyl synthase. Free apiose was reduced to apiitol which was oxidized with periodic acid to glycolic acid. In both cases oxidation of glycolic acid with glycollate oxidase from spinach to glyoxalic acid released all the tritium into the water. This result proves that during the hydride shift from UDP-[4-^{3}H]GlcUA tritium is transfered only to the *pro-R* position at C-3′ of apiose and that inversion of configuration occurs at C-4 of apiose in the synthase reaction.

3. When the UDP-Api/UDP-Xyl synthase reaction was carried out in a $^{3}H^{1}HO/^{2}H_{2}O$ mixture, a total of 1.5 mol ^{3}H per mol apiose was incorporated with correction made for the product isotope effect. By degradation of apiose it was proved that 1 mol ^{3}H was located at C-4 and 0.5 mol ^{3}H per mol sugar at C-3′ of apiose.

4. After addition of $NaB^{3}H_{4}$ to the enzyme incubation with UDP-D-[U-^{14}C]GlcUA and hydrolysis of the nucleotide sugars, arabinose, xylose and glucuronic acid with a constant $^{3}H/^{14}C$ ratio were isolated after extensive purification. It was shown by degradation of xylose that over 90% of the tritium was located at C-4 of this sugar. These results prove the existence of a 4-keto intermediate and the formation of UDP-4-keto-glucuronic acid as an intermediate in the decarboxylation step.

5. The results presented can best be explained by an aldolase type mechanism for the UDP-Api/UDP-Xyl synthase reaction.

The enzyme UDP-apiose/UDP-xylose synthase catalyzes the NAD^{+}-dependent conversion of UDP-D-glucuronic acid to UDP-D-apiose and UDP-D-xylose [1]. Despite the approximately 1000-fold purification of the enzyme from cell suspension cultures of parsley (*Petroselinum hortense* Hoffm.) the enzymatic activities for apiose and xylose synthesis were not separated [2]. In previous work on the mechanism of the rearrangement of the glucuronic acid moiety to the branched pentose D-apiose (Fig. 1, II), it was shown that the branched carbon of this sugar (C-3′) originates from C-3 of D-glucuronic acid [3] and that a hydride shift from C-4 of UDP-GlcUA to C-3′ of apiose occurs during the reaction [4]. In order to gain further insight into this interest-

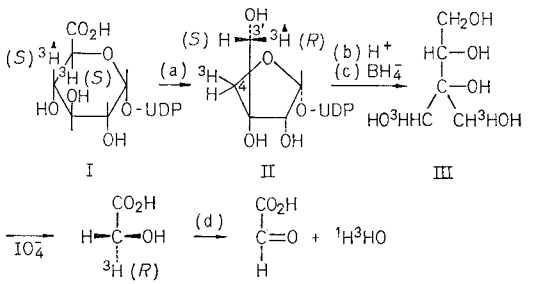

Fig. 1. *Determination of stereochemistry in the conversion of UDP-[4,5-$^{3}H_{2}$]GlcUA to UDP-[3′,4-$^{3}H_{2}$]-Api.* (a) UDP-Api/UDP-Xyl synthase; (d) glycollate oxidase and catalase

Abbreviations. UDP-Api, uridine diphospho-D-apiose; UDP-Xyl, uridine diphospho-D-xylose; UDP-GlcUA, uridine diphospho-D-glucuronic acid; PPO, 2,5-diphenyloxazole; apiin, 7-*O*-[β-D-apiofuranosyl-(1 → 2)-β-D-glucosyl]-5,7,4′-trihydroxyflavone.

Enzymes. UDP-L-arabinose 4-epimerase or UDP-arabinose epimerase (EC 5.1.3.5); UDP-glucuronate carboxy-lyase or UDP-glucuronate decarboxylase (EC 4.1.1.35); glycollate: oxygen oxidoreductase or glycollate oxidase (EC 1.1.3.1); hydrogen peroxide: hydroxgen peroxide oxidoreductase or catalase (EC 1.11.1.6).

ing rearrangement we have now investigated the following: (a) the question whether UDP-D-xylose can be converted to UDP-D-apiose; (b) the absolute configuration of tritium at C-3′ and C-4 of apiose originating from UDP-[4-^{3}H]GlcUA and UDP-[5-^{3}H]GlcUA, respectively; (c) the incorporation of tritons from the medium into apiose during the synthase reaction in $^3H^1HO/^2H_2O$ and (d) the tritiated products which are formed upon addition of sodium boro[^{3}H]hydride to the synthase incubation.

MATERIALS AND METHODS

Materials

D-[3-^{14}C]Glucose (10 mCi/mmol) was obtained from New England Nuclear Corp. (Boston). All other labeled compounds were purchased from Radiochemical Centre (Amersham). These included: D-[4-^{3}H]glucose (7.1 Ci/mmol), D-[5-^{3}H]glucose (1 Ci/mmol), UDP-D-[U-^{14}C]GlcUA (287—319 mCi/mmol), NaB^3H_4 (7.2 Ci/mmol and $^1H^3HO$ (5 Ci/mmol). ^2H_2O with 99.9% purity was obtained from Merck AG (Darmstadt). Catalase was purchased from Boehringer Mannheim GmbH. Di-*O*-isopropylidene apiose was obtained from Pfanstiehl Labs. (Wankegan, Illinois).

Chromatography

Schleicher & Schüll paper No 2043 b or Whatman 3 MM was used for chromatography. For preparative purposes the paper was prewashed with 0.01 M EDTA, 10% acetic acid, water and methanol. The following solvent systems were used (the ratios are all by vol.): (1) 15% acetic acid; (2) ethylacetate–pyridine—water (8:2:1); (3) ethanol—1 M ammonium acetate, pH 3.8 (5:2); (4) *n*-butanol—acetic acid—water, upper phase (4:1:5); (5) diethylether —88%formic acid—water (5:2:1); (6) ethylacetate—acetic acid—water (3:1:3); (7) ethylmethylketone—acetic acid—sat. boric acid solution—water (8:1:1:1); (8) *n*-butanol—ethanol—water (4:1:1); (9) *n*-butanol—pyridine—water (6:4:3); (10) *iso*-propanol—pyridine—acetic acid—water (8:8:1:4); (11) *n*-butanol—ethanol—water (5:2:3); (12) ethylacetate—pyridine—water (2:1:2); (13) ethylmethylketone—acetic acid—water (8:1:4); (14) *n*-butanol—ethanol–water (5:2:2); (15) *iso*-propanol—ethanol—water (4:3:2); (16) *n*-butanol—acetic acid—water (2:1:1); (17) *iso*-propanol—pyridine—water (3:1:2); (18) ethylmethylketone—pyridine—water (3:1:3); (19) *iso*-propanol—ethylacetate—water (5:3:3); (20) ethylacetate—pyridine—water (3:4:4). Reference sugars were detected with aniline phthalate.

Radioactivity Measurements

A Beckman model LS-233 scintillation spectrometer was used. For non-aqueous solutions and paper chromatograms a solution consisting of 0.5% PPO in toluene was used. Aqueous solutions were counted in 10% naphthalene and 0.5% PPO in dioxan. Corrections were made for counting efficiency and for ^{14}C contribution to the tritium channel by use of [^{14}C]toluene and [^{3}H]toluene as internal standards. Paper strips were scanned with the scanner LB 280 from Berthold-Frieseke GmbH (Karlsruhe).

UDP-Api/UDP-Xyl Synthase

The enzyme used in all experiments described was purified from cell suspension cultures of parsley according to the published procedure [2] with the exception that the hydroxyapatite step was omitted. Purification was about 150—200 fold.

Enzymatic Production of Labeled UDP-GlcUA

UDP-D-[4-^{3}H]GlcUA and UDP-D-[5-^{3}H]GlcUA were prepared from the correspondingly labeled glucose as described previously [4]. In the published procedure it was erroneously stated that 2.5 μmol UDP-Na_3H were used in the incubation. This should be corrected to read UTP-Na_3H.

Enzymatic Production of UDP-D-[U-^{14}C]Xyl

For the incubation the UDP-glucuronate carboxy-lyase from cell suspension cultures of parsley which had been separated on a DEAE-cellulose column from the UDP-Api/UDP-Xyl synthase was used [25]. UDP-D-[U-^{14}C]Xyl was produced from UDP-D-[U-^{14}C]GlcUA in an incubation mixture consisting of the following: 500 μl UDP-D-[U-^{14}C]-GlcUA (1.87×10^6 dis./min, 2.95 nmol), 300 μl 10 mM dithioerythritol, 500 μl 0.2 M sodium acetate buffer pH 5.5 and 2000 μl enzyme (approx. 40 mg protein). The mixture was incubated at 30 °C for 31 h; after 13 h another 100 μl of enzyme was added. The reaction mixture was chromatographed on paper with solvent system (3) for 39 h. The tank was pre-equilibrated with ethanol—water (5:2, v/v). The UDP-Xyl zone ($R_{UDP\text{-}GlcUA}$ 0.97) was first treated with absolute ethanol at 20 °C to remove ammonium acetate, and the labeled product was then eluted with water. After a second chromatography with solvent system (3) the substance was pure. Yield: 4.6×10^5 dis./min (725 pmol) UDP-D-[U-^{14}C]Xyl (25%).

Enzymatic Production of [3′-^{3}H]Apiose and [4-^{3}H]Apiose and Reduction to Apitol

UDP-[3′-^{3}H]Api and UDP-[4-^{3}H]Api were produced from UDP-D-[4-^{3}G]GlcUA and UDP-D-[5-^{3}H]-GlcUA, respectively, in the following incubation

mixture: 500 µl 0.5 M Tris-HCl pH 8.2, 200 µl 25 mM NAD, 150 µl 10 mM dithioerythritol, 450 µl of the labeled UDP-GlcUA (3.18 nmol, 5×10^7 dis./min) and 200 µl enzyme (60 µg/ml). After incubation for 10 h at 30 °C another 150 µl NAD, 100 µl dithioerythritol and 500 µl enzyme were added and the incubation was continued for 14 h. At the end of this period the nucleotide sugars were hydrolysed by adding trifluoroacetic acid to 10% concentration and heating the solution to 95 °C for 30 min. The sugars were then separated by chromatography twice on paper with solvent system (2) for 12 h. Yield of apiose 13.7×10^6 dis./min (870 pmol).

Part of the labeled apiose (6.85×10^6 dis./min) was then diluted with 50 mg unlabeled apiose and reduced with $NaBH_4$ to apiitol [18]. Apiitol was isolated by chromatography on Dowex 1-acetate and further purified by paper chromatography with solvent system [4]. Yield of apiitol 6.52×10^6 dis./min (317 µmol).

Periodate Oxidation of Apitol and Oxidation of Glycolic Acid

The periodate oxidation of apitol was carried out as described in the literature [18]. The glycolic acid from the eluate of the Dowex 1-acetate column was purified on paper with solvent system (5). Glycolic acid (6.6×10^5 dis./min, 62.8 µmol) was then oxidized with glycollate oxidase according to a published procedure [10]. The enzyme from spinach had been purified about 90-fold [14].

Incubation in $^1H\ ^3HO/^2H_2O$

The incubation mixture contained: 1070 µg (1.6 µmol) NAD, 430 µg (2.9 µmol) dithioerythritol, 16.8 nmol (10.6×10^6 dis./min) UDP-[U-^{14}C]GlcUA, 180 µl (0.9 Ci) $^3H\ ^1HO$ and freeze-dried enzyme (250 µg protein) dissolved in 300 µl ^2H_2O. Freeze-drying was carried out with the enzyme in 0.1 M Tris-HCl buffer pH 8.0. It was therefore not necessary to add further buffer to the incubation. After incubation for 3.5 h at 30 °C 300 µl was withdrawn for the apiosyltransferase reaction (see below). The rest of the mixture was incubated for another 19 h. At the end of this period 20 µl acetic acid was added and the water distilled into a small trap by freeze-drying; 3 ml water was then added to the residue and the water was again removed by freeze-drying. This procedure was repeated another 3 times. To the residue was then added 1 ml water and 140 µg each of arabinose, xylose and apiose. After addition of 200 µl trifluoroacetic acid the mixture was kept for 30 min at 95 °C. The free sugars were purified by paper chromatography with solvent systems (2), (4) and (6) until they had reached a constant $^3H/^{14}C$ ratio.

Apiosyltransferase Reaction

The transfer of apiose from UDP-Api to 7-*O*-glucosylapigenin was carried out as described previously [4] with the following modifications. The transferase was obtained from cell suspension cultures of parsley and purified according to the published procedure including the ammonium sulfate precipitation step [13]. Apiin was purified by paper chromatography with solvent system (1), recrystallization from ethanol—water and chromatography on Sephadex LH 20 with methanol.

Incubation with Addition of NaB^3H_4

The incubation mixture contained 800 µg NAD, 300 µg dithioerythritol, 8 nmol (5.88×10^6 dis./min) UDP-D-[U-^{14}C]GlcUA, 50 µl 0.5 M Tris-HCl pH 8.0 and 300 µl enzyme (1.25 mg protein). Incubations were carried out for 3 min at 30 °C. The reaction mixture was cooled to 0 °C for 2 min and then 50 µl of a solution of NaB^3H_4 (1.4 µmol, 1.1×10^{10} dis./min) were added. Incubations were kept at room temperature for another 22 h. After hydrolysis of the nucleotide sugars with trifluoroacetic acid as described above, the pentoses were purified by chromatography with solvent systems (2, 4, 6, 8, 9, 10, 12, 14, 15, 16, 19) and GlcUA with solvent systems (2, 6—11, 13, 15, 17, 18, 20).

RESULTS

Can UDP-Xyl be converted to UDP-Api

Though dependence of the reaction rate and of the apiose/xylose ratio on time and the effect of NH_4^+ ions on apiose and xylose formation are compatible only with a parallel synthesis of apiose and xylose [5], a possible conversion of UDP-Xyl to UDP-Api (II) *via* the hypothetical 4-keto intermediate V (Fig. 2) had not yet been excluded. Therefore UDP-[U-^{14}C]Xyl was incubated in the enzyme assay [6] with the synthase for 5 h and the sugars were separated with solvent system (2). The results are shown in Table 1. No radioactivity was detected in apiose under these conditions.

Absolute Configuration at C-3′ and C-4 of Apiose

In order to determine which of the two diastereotopic [7] hydrogens of the prochiral methylene (C-3′) of apiose becomes labeled in the hydride shift from C-4 of UDP-GlcUA, [3′-^{3}H]apiose obtained from UDP-[4-^{3}H]GlcUA [4] was reduced with sodium borohydride to apiitol (Fig. 1, III). [2-^{3}H]Glycolic acid obtained by periodate oxidation of apiitol was then oxidized with glycollate oxidase from spinach [14], which is specific for the *pro-R* hydrogen [8, 11]. The reaction products were separated on Dowex 1-acetate with 4 N acetic acid [9]. The results presented

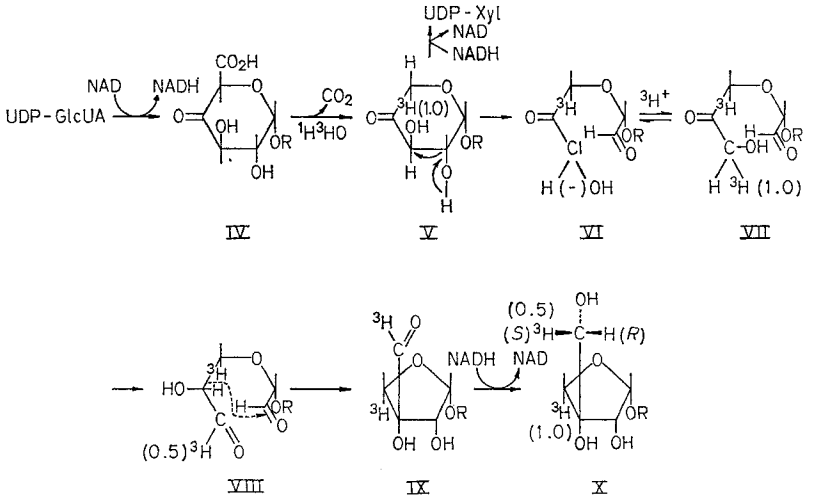

Fig. 2. *Incorporation of tritium during the UDP-Api/UDP-Xyl synthase reaction in $^3H^1HO$.* Numbers in brackets give tritium atoms incorporated corrected for product isotope effect (see text). Compounds IV–IX are enzyme-bound intermediates. R = UDP

Table 1. *Incubation of UDP-Api/UDP-Xyl synthase with UDP-[U-^{14}C]Xyl*
Incubations were carried out for 5 h in the enzyme assay [6] with 37400 dis./min (58.7 pmol) UDP-[U-^{14}C]GlcUA or 30700 dis./min (48.5 pmol) UDP-[U-^{14}C]Xyl

Substrate	Incubation	Apiose	Xylose
		counts/min	
UDP-[U-^{14}C]GlcUA	complete	2030	6520
	control[a]	90	145
UDP-[U-^{14}C]Xyl	complete	0	11200
	control[a]	0	11030

[a] With boiled enzyme (3 min at 100 °C).

Table 2. *Oxidation of [^{3}H]glycolic acid with glycollate oxidase*
In experiment I 6.35 μmol (66000 dis./min) of glycolic acid was incubated for 14 h and the reaction products separated as described under Methods. Yield of glyoxalic acid 4.6 μmol (73%). In experiment II 4.13 μmol (60700 dis./min) yielded 2.8 μmol (68%)

Substrate	Unreacted glycolic acid	Glyoxalic acid	Water
		dis/min	
I Glycolic acid from [3′-^{3}H]apiose	27800	0	36200
Control[a]	61000	0	0
II Glycolic acid from [4-^{3}H]apiose	28530	0	32100
Control[a]	60250	0	0

[a] With boiled enzyme (3 min at 100 °C).

in Table 2 (experiment I) show that the glycolic acid loses all its label upon oxidation with glycollate oxidase. This result proves that in the hydride shift from UDP-[4-^{3}H]GlcUA, tritium is transfered only to the *pro-R* position at C-3′ of apiose.

Schutzbach and Feingold [10] had shown that during the conversion of UDP-[5-^{3}H]GlcUA to UDP-[5-^{3}H]Xyl by UDP-glucuronate carboxylyase from wheat germ the configuration at C-5 changed from *S* to *R*. It was of interest to see whether inversion of configuration also occurs at C-4 of apiose in the UDP-Api/UDP-Xyl synthase reaction. With UDP-[5-^{3}H]GlcUA as substrate the same series of reactions as described above for determination of the stereochemistry of the hydride shift was repeated. If inversion of configuration at C-4 of apiose takes places [2*R*-^{3}H]glycolic acid would be obtained upon periodate oxidation of the corresponding apiitol. This was indeed the case, as is shown by the results in Table 2 (experiment II). The stereochemical course of the reaction during the conversion of UDP-[4,5-$^3H_2$]GlcUA to UDP-[3′,4-$^3H_2$]Api and the determination of the stereochemistry is summarized in Fig. 1.

Incorporation of Protons (Tritons) into Apiose during the UDP-Api/UDP-Xyl Synthase Reaction

It has been shown previously that during the conversion of UDP-[3-^{3}H]GlcUA to UDP-[3′-^{3}H]Api about 50% of the tritium is lost, whereas with UDP-[4-^{3}H]GlcUA as substrate a loss of only about 12% tritium occurs, which could be explained by a kinetic isotope effect [4]. If the 50% loss of tritium is due to labilization of tritium in an intermediate, tritons from the medium should be incorporated into

Table 3. *Incorporation of tritium into sugars during the UDP-Api/UDP-Xyl synthase reaction in $^3H^1HO/^2H_2O$*

Substance	$^{14}C/^3H$	^3H incorporated				
		without pie[a]	with pie[a] of 1.9 ± 0.1 [12]	with pie[a] of 2.02 ± 0.1	with D-xylose as standard	with L-arabinose as standard
		mol/mol				
D-Xylose	45.1	0.49	0.93	1.0	1.0	1.0
L-Arabinose	45.5—47.4	0.47—0.49	0.90—0.93	1.0	1.0	1.0
D-Apiose	27.9—28.8	0.75—0.78	1.42—1.48	1.54	1.53—1.59	1.53—1.66
Formaldehyde-dimedone (C-3′ of apiose)	17.6	0.24	0.46	0.49	—	—

[a] Product isotope effect.

C-3′ of apiose when the enzymatic reaction is carried out in tritiated water.

A product isotope effect of 7.1 has been determined for tritium as a tracer in protium oxide, whereas for tritium as a tracer in deuterium oxide the product isotope effect is only 1.9 [12]. It is therefore advantageous to carry out the incubation in ^2H_2O. Since theoretically 1 triton must be incorporated at C-4 of apiose during the decarboxylation of UDP-GlcUA (see above) and 0.5 triton was expected to be incorporated at C-3′, the tritium activity in these two carbon atoms had to be determined separately. The experiment was therefore carried out as follows. UDP-[U-^{14}C]GlcUA was incubated with the synthase in ^2H_2O containing 525 mCi tritium for 3.5 h. The incubation mixture was then divided into two portions. In one portion apiose was transferred from UDP-Api to 7-*O*-(β-glucosyl)-apigenin with an apiosyltransferase from parsley cell cultures [13] as has been described previously [4]. The resulting apiin (7-*O*-[β-D-apiofuranosyl-(1 → 2)-β-D-glucosyl]-5,7,4′-trihydroxyflavone) was purified and then oxidized with periodate in sodium bicarbonate to yield the C-3′ of apiose as formaldehyde, which was trapped as the dimedone derivative [4].

The second portion of the incubation mixture was kept for another 19 h at room temperature. After hydrolysis of the nucleotide sugars with acetic acid to D-apiose, D-xylose and L-arabinose (the synthase in this experiment contained a small activity of UDP-arabinose epimerase) the sugars were purified by paper chromatography is solvent systems (2, 4, 6) and again (2) until they had reached a constant $^{14}C/^3H$ ratio. The results are shown in Table 3.

With no correction for a product isotope effect D-apiose contained 0.75—0.78 mol ^3H, D-xylose 0.49 mol ^3H and L-arabinose 0.47—0.49 mol ^3H/mol sugar. Taking xylose and arabinose as a standard which must incorporate 1 mol ^3H/mol sugar during decarboxylation [10] it was calculated that apiose incorporated respectively 1.53—1.59 and 1.53—1.66 mol ^3H/mol. Taking into account a product isotope effect for tritium in ^2H_2O of 1.9 ± 0.1, as determined by Gold and Kessick [12], apiose contains 1.42—1.48 mol ^3H, xylose 0.93 mol ^3H and arabinose 0.9—0.93 mol ^3H/mol sugar. If the value for xylose and arabinose is assumed to be 1.0 mol ^3H/mol sugar, the product isotope effect in our system is 2.02 ± 0.10. The $^{14}C/^3H$ ratio found in formaldehyde proves that about 0.5 mol ^3H/mol apiose is incorporated at C-3′ during the synthase reaction in $^3H^1HO$. This value is in excellent agreement with the 50% loss of tritium in the synthase reaction with UDP-[3-^{3}H]GlcUA as substrate (see above).

Proof for the Existence of UDP-4-keto-GlcUA and UDP-4-keto-pentose as Intermediates in the UDP-Api/UDP-Xyl Synthase Reaction

Reduction with NaB^3H_4 has been succesfully applied to provide direct evidence for the existence of enzyme-bound 4-keto-sugar intermediates [15, 16]. Since a 4-keto intermediate has been proposed for the UDP-Api/UDP-Xyl synthase reaction [17, 18], this method was now used in order to trap such an intermediate.

UDP-[U-^{14}C]GlcUA (7.98 nmol, 5.88×10^6 dis./min) was incubated for 3 min with 1.25 mg synthase. At the end of this period 1.4 μmol NaB^3H_4 (7.2 Ci/nmol) was added at 0 °C and the reaction mixture was kept another 22 h at room temperature. Controls contained denatured protein or no NaB^3H_4. After precipitation of the protein with trichloroacetic acid and hydrolysis of the nucleotide sugars, the sugars were purified by 13 chromatographic purifications on paper till they had reached a constant $^3H/^{14}C$ ratio (Table 4). It can be seen from Table 4 that GlcUA, arabinose and xylose reached a constant $^3H/^{14}C$ ratio, whereas apiose from the normal incubation and GlcUA from the control incubation with heat-denatured enzyme contained only insignificant amounts of tritium or none at all.

The tritium in xylose was localized in the following way. After dilution with unlabelled xylose the phenylosazone was prepared and oxidized with

Table 4. *$^3H/^{14}C$ ratio in sugars after addition of NaB^3H_4 to the UDP-Api/UDP-Xyl synthase reaction*
Incubations were carried out with UDP-[U-^{14}C]GlcUA as described in the text

Number of purification steps	$^3H/^{14}C$ in:				
	GlcUA	GlcUA (control)[a]	Arabinose	Xylose	Apiose
5	8.0	2.2	1.4	1.2	0.1
10	0.72	0.27	0.43	0.32	0
12	0.57	0.08	0.36	0.36	—
13	0.65	0.04	0.40	0.35	—

[a] With boiled enzyme (3 min at 100 °C).

Table 5. *Distribution of tritium activity in periodate oxidation products of* D-*xylose-phenylosazone*

Compound	Hydrogen atoms bound to designated carbon atoms of xylose	Specific activity
		dis × min^{-1} × μmol^{-1}
Xylose	1, 2, 3, 4, 5	111
Xylose phenylosazone	1, 3, 4, 5	114
Mesoxalaldehyde 1,2-bis (phenylhydrazone)	1, 3	3
p-Bromphenacyl formate	4	102
Methylene bis(dimedone)	5	10

periodic acid to yield mesoxalaldehyde 1,2-bis(phenylhydrazone) from C-1, C-2 and C-3, formic acid from C-4, and formaldehyde from C-5 of xylose [10,15]. Formaldehyde was isolated as the dimedone derivative and formic acid as *p*-bromophenacyl formate [19]. The results presented in Table 5 prove that about 90% of the tritium activity is located at C-4 of xylose. Whether the small amount of tritium present in the formaldehyde (C-5) is of any significance is unknown. The methylene bisdimedone might still have contained some radioactive impurity.

DISCUSSION

The finding that incubation of UDP-[U-^{14}C]Xyl with the UDP-Api/UDP-Xyl synthase free of UDP-arabinose epimerase does not lead to incorporation of label into UDP-Api is consistent with earlier results indicating a parallel synthesis of UDP-Api and UDP-Xyl [5]. Since the 4-ketopentose V (Fig. 2) is very probably a common intermediate for apiose and xylose synthesis, the reduction of V to UDP-Xyl is apparently an irreversible reaction, at least under the conditions of the enzyme assay.

In the hydride shift from UDP-[4-^{3}H]GlcUA tritium is transferred stereospecifically to the *pro-R* position at C-3′ of apiose (Fig. 1). In this connection it is interesting to note that the hydrogen introduced or removed by the action of liver alcohol dehydrogenase on a wide variety of substrates is also the one with the *pro-R* orientation [20]. The inversion of configuration at C-4 of apiose (Fig. 1) is in agreement with the stereochemical course of the UDP-glucuronate carboxylyase reaction at C-5 [10,21].

The incorporation of 0.5 mol ^{3}H/mol sugar at C-3′ of apiose during the synthase reaction in ^{3}H ^{1}HO and the loss of 50% tritium during this reaction with UDP-[3-^{3}H]GlcUA as substrate can respectively be explained only if this hydrogen (tritium) atom becomes equivalent to another hydrogen (tritium) during the reaction. While this postulate is not consistent with the concerted mechanism for the ring contraction leading to apiose [17], the aldolase-type mechanism proposed by Picken and Mendicino [18] could explain this observation (Fig. 2). The carbanion VI assumed to be formed by aldol cleavage of V would incorporate one tritium atom leading to VII. Under the assumption that the two hydrogen atoms at the primary hydroxyl group in VII cannot be differentiated by the enzyme and that no kinetic effect occurs, 50% of the tritium would be expected to be lost in the subsequent isomerization step leading to VIII. It has to be further assumed that the isomerization from VII to VIII is practically irreversible under normal conditions. One half of the tritium incorporation from the medium or originally present at C-3 of UDP-GlcUA appears finally in the *pro-S* position at C-3′ of apiose (see above).

The isolation of tritium-labeled glucuronic acid, arabinose and xylose after addition of NaB^3H_4 to the enzyme incubation (Table 4) and the localization of tritium at C-4 of D-xylose (Table 5) proves the existence of the postulated 4-keto intermediate V (Fig. 2) and the formation of UDP-4-keto-GlcUA (IV) as an intermediate in the decarboxylation step [22]. A similar experiment to demonstrate the existence of enzyme-bound dTDP-4-keto-D-glucose in the dTDP-D-glucose oxidoreductase reaction [16] has been criticized because the quantity of the [4-^{3}H]galactose isolated far exceeded the quantity of oxidoreductase present [23]. In our case the quantitative relationship between enzyme present and tritiated products formed can be estimated as follows. The incubation contained 1.25 mg enzyme with a purity of about 12%. With a molecular weight of about 100000 [2] this amounts to about 1.4 nmol enzyme. The NaB^3H_4 had an activity of 4000 dis. × min^{-1} × pmol tritium^{-1}. The xylose contained 134500 dis./min tritium which corresponds to 34 pmol tritium. The sum of the tritiated sugars (GlcUA + xylose + arabinose) was 625000 dis./min, corresponding to 156 pmol sugar. The ratio of enzyme to tritiated sugars is therefore 9:1. The calculation is based on the tritium activity of the NaB^3H_4 given by the supplier which is probably not very accurate.

Since no incorporation of tritium into apiose was found, the existence of a free aldehydo intermediate (Fig. 2, IX) in apiose biosynthesis is very unlikely.

In analogy to the reaction mechanism of type I aldolases [24] the keto intermediates IV—VII could be bound as a Schiff base to lysine. In this case reduction with borohydride would lead to ^{14}C- and ^{3}H-labeled protein. This was indeed the case. However, this type of experiment has to be repeated with a pure enzyme before the existence of a Schiff base intermediate can be proved.

In summary, all results obtained so far are consistent with the mechanism outlined in Fig. 2, with the aldehyde group of IX in an enzyme-bound form. To gain further insight into this interesting sequence of reactions the properties of the enzyme (number of subunits and binding sites *etc.*) must be studied.

This work was supported by *Deutsche Forschungsgemeinschaft*. D. Baron thanks the *Studienstiftung des Deutschen Volkes* for a *Promotionsstipendium*.

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