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# Biosynthesis of Vitamin E and of the Plastoquinones in Chloroplasts: Steric Course of the Decarboxylation

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Samples of (3*R*)- and (3*S*)-4′hydroxyphenyl[3-$^2H_1$, 3-$^3H$]pyruvate were prepared by taking advantage of the known stereospecificity of phenylpyruvate keto–enol isomerase (tautomerase). 4′-Hydroxyphenyl[3-$^{14}C$]pyruvate was obtained by the action of L-amino acid oxidase on DL-[3-$^{14}C$]tyrosine, whereas a simple base-catalyzed exchange procedure yielded samples of 4′-hydroxyphenyl[3-$^3H$]- and 4′-hydroxyphenyl[3-$^2H_2$]pyruvate. All labeled samples were converted *in situ* into the corresponding homogentisic acids on 4′-hydroxyphenylpyruvate dioxygenase that is known to catalyze the migration of the acetate side chain with retention of configuration. The isolated doubly labeled homogentisic acids were incubated with chloroplasts from *Raphanus sativus* cv. *saxa* Treib, and from the lipophilic products a fraction containing *inter alia* tocopherol, tocoquinone, and plastoquinone was obtained by chromatographic procedures. The incorporation of radioactivity was between 0.5 and 11% based on homogentisate. Reductive acetylation of the quinones yielded crystalline diacetylhydroquinones, which were submitted to Kuhn–Roth degradation. The radioactive acetate samples thus obtained were analyzed for chirality by an enzymatic procedure previously published. (2*R*)-[2-$^2H_1$, 2-$^3H$]Homogentisate gave mainly (*S*)-acetate, whereas (2*S*)-[2-$^2H_1$, 2-$^3H$]homogentisate was converted mainly into (*R*)-acetate. It is concluded that the decarboxylation of the side chain occurred with stereochemical retention during the biosynthetic process. 

## INTRODUCTION

Homogentisic acid (**1**) is a precursor of α-tocopherol (**2**) (vitamin E), plastohydroquinone (**3**), and their oxidized forms, tocoquinone and plastoquinone [Eq. 1] (*1*, *2*).

In addition to the introduction of an isoprenoid side chain and of some methyl groups from adenosyl methionine one methyl group (labeled with Δ) is generated by decarboxylation of the original acetic acid side chain of homogentisic acid (**1**). We decided to determine the steric course of this decarboxylation by using stereospecifically labeled ([$^2H$] and [$^3H$]) homogentisates as precursors. Previous work in our laboratory (*3*, *4*) paved the way for such a labeling by taking advantage of the stereospecificity of two well-characterized enzymes, the phenylpyruvate keto–enol isomerase (tautomerase) and the 4′-hydroxyphenylpyruvate



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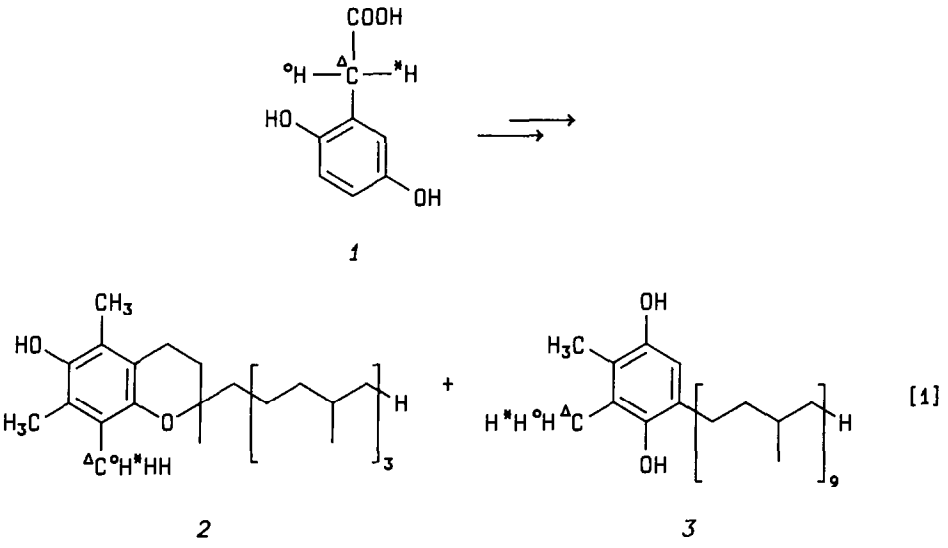
dioxygenase. In the following we describe the experiments leading to the research goal mentioned above.




1 2 3 [1]

## EXPERIMENTAL PROCEDURES

### *General Methods and Equipment*

Melting points were determined using a Büchi apparatus and are uncorrected. $^1$H NMR spectra were recorded on a Bruker WH 90 or WM 250 spectrometer. Chemical shifts ($\delta$) are reported in ppm relative to tetramethylsilane, coupling constants ($J$) in Hz.

For uv spectra a Perkin–Elmer 550 A spectrophotometer was used.

Radioactivity ([$^3$H] + [$^{14}$C]) was measured with a Searle Nuclear Chicago Division Isocap 300 liquid scintillation spectrometer. One liter of the liquid scintillation counting fluid consisted of 833 ml 1,4-dioxane (AR grade), 167 ml ethyleneglycol monoethylether, 50 g naphthalene, 4 g 2,5-diphenyloxazole, and 0.1 g 1,4-bis[2-(5-phenyloxazolyl)]benzene (both oxazole fluors were obtained from Koch-Light Laboratories Ltd, Colnbrook, Bucks, Great Britain). The samples were dissolved in 10 ml of this solution in a "polyvial" from Zinsser (Frankfurt, Federal Republic of Germany). Counting efficiencies of 40–45% for tritium and 80–90% for $^{14}$C were obtained in simply labeled samples. The corresponding values for doubly labeled samples were 35–42% for $^3$H and 44–45% for $^{14}$C.

Radioactivity on thin-layer plates was detected by a LB 2721 scanner (Bertold Ltd, Wildbad, Federal Republic of Germany).

For the assay and isolation of 4′-hydroxyphenylpyruvate dioxygenase the same equipment was used as described by Leinberger *et al.* (*4*). The illumination of the chloroplast suspensions occurred with an Attralux bulb through an infrared filter.

*Materials*

4′-Hydroxyphenylpyruvate dioxygenase (EC 1.13.11.27) was isolated from beef liver according to the method of Leinberger *et al.* (*4*) except that commercial hydroxylapatite was used. Its specific activity was about 0.027 U/mg.

Malate synthase (EC 4.1.3.2) from yeast was isolated according to Ref. (*5*). The enzyme preparation had a specific activity of 0.65 U/mg. Phenylpyruvate keto–enol isomerase or tautomerase (EC 5.3.2.1), 1-2 U/mg; phosphotransacetylase, phosphate acetyltransferase (EC 2.3.1.8), 200 U/mg; 4′-hydroxyphenylpyruvate (Grade I); and homogentisate were purchased from Sigma. L-Amino acid oxidase (EC 11.4.3.2) from *Crotalus durissus* (7 U/mg), acetate kinase (EC 2.7.2.1) from *Escherichia coli* (200 U/mg), catalase (EC 1.11.1.6) from beef liver (65,000 U/mg), malate dehydrogenase (EC 1.1.1.37) from pig heart (1200 U/mg), fumarase (EC 4.2.1.2) from pig heart (350 U/mg), coenzyme A (free acid, Grade I), $NAD^+$ (free acid, Grade II), ATP (disodium salt), and glutathione (red.) were products of Boehringer & Soehne GmbH (Mannheim). Hydroxylapatite "spheric," deuterium oxide (99–75%), 2,6-dichlorophenolindophenol (sodium salt), and phenylmethanesulfonylfluoride were purchased from Merck AG (Darmstadt). SP-Sephadex C-50 and Sephadex G-25 were from Pharmacia Fine Chemicals (Freiburg), while cellulose DE-23 from Whatman.

*Radiochemicals.* DL-[3-$^{14}$C]Tyrosine acetate was from Zinsser Analytik GmbH (Frankfurt), and $^3H_2O$ (3 Ci/ml) from Amersham Buchler GmbH & Co. KG (Braunschweig). Bakers yeast was a present of the firm Fala (Strasbourg, France).

*Isotopically Labeled Homogentisates* (**4**)

Sodium 4′-hydroxyphenylpyruvate (200 mg, 990 mol) in deuterium oxide (50 ml) was stirred at 25°C and p$^2$H 9.3 (adjusted with $NaO^2H$) for 5 h. After lyophilization the residue was again incubated in fresh deuterium oxide (25 ml) for 2 h. Neutralization (aqueous HCl) to p$^2$H 7 was followed by lyophilization, which yielded 4′-hydroxyphenyl[3-$^2H_2$]pyruvate as a white powder. The deuterium isotope shift in the $^1$H NMR spectrum was used for deuterium analysis and showed 0.1–1.85% unlabeled, 1.7–5% [$^2H_1$], and 93–98% [$^2H_2$] methylene groups. Integration in the aromatic region indicated that about one-third of the 3′-protons were exchanged against deuterium.

Sodium(3*RS*)-4′-hydroxyphenyl[3-$^3$H]pyruvate was prepared by an analogous method. The unlabeled compound (40.2 mg, 199 $\mu$mol) was incubated at 25°C and pH 9.3 in 1 ml tritiated water (3 Ci). Neutralization with a few drops of 0.1 M HCl was followed by bulb-to-bulb lyophilization. The latter was repeated three times with 0.5 ml added unlabeled water in order to remove all labile tritium. The resulting product was chromatographically pure (thin layer; *n*-butanol/water/formic acid, 70/25/5; $R_f = 0.72$) and exhibited a total radioactivity of 710 $\mu$Ci ($1.59 \times 10^9$ dpm $^3$H). It was stored at −20°C.

A commercial solution (0.72 ml) of DL-[3-$^{14}$C]tyrosine acetate (1.2 $\mu$mol, 60 $\mu$Ci) was lyophilized and dissolved in $O_2$-saturated potassium phosphate buffer (0.65 ml, 50 mM, pH 6.8). Solutions of catalase (0.01 ml, 0.2 mg, 65,000 U/mg) and of L-amino acid oxidase from snake venom (0.04 ml, 0.4 mg, 7 U/mg) were then added

to a total volume of 1.06 ml. After incubation (60 min, 25°C) the mixture was immediately chromatographed (Dowex 50 W × 8 column, 1.1 × 12 cm, $H^+$-form, 200–400 mesh). 4′-Hydroxyphenyl[3-$^{14}$C]pyruvic acid was eluted with distilled water, and each tube receiving the fractions (1 ml) contained sodium phosphate buffer (75 μl, 400 mM, pH 7.4). The unreacted D-tyrosine was eluted from the column with an HCl gradient (0.15 → 0.04 M). The fractions (50 ml) containing 4′-hydroxyphenyl[3-$^{14}$C]pyruvate (0.56 μmol, 62.1 × $10^6$ dpm, 28 μCi) were lyophilized and then the solid residue was chromatographed on a silica gel plate (*n*-butanol/water/formic acid, 70/25/5). The radioactivity was localized at an $R_f$ value of 0.70 coinciding with that of authentic 4′-hydroxyphenylpyruvate. The $R_f$ value for tyrosine was 0.38 in the same system.

To a solution of sodium 4′-hydroxyphenyl[3-$^2$H]pyruvate (17.5 mg, 86.5 μmol) in deuterated sodium phosphate buffer (42 ml, 200 mM, p$^2$H 6.2) tritiated water (1 ml, 3 Ci) and tautomerase (10 U, 1–2 U/mg) were added and the mixture was incubated in a closed vessel for 90 min at 25°C. After bulb-to-bulb lyophilization the recovered tritiated water was used to carry out the enzymatic tritiation of another portion of 17.5 mg 4′-hydroxyphenyl[3-$^2H_2$]pyruvate in the same manner as just described. (The comparatively large volume of 43 ml was necessary to avoid substrate inhibition of tautomerase.) The combined products from two incubations were dissolved in deuterated sodium phosphate buffer (85 ml, 200 mM, p$^2$H 6.2). To this radioactive solution (35 × $10^6$ dpm $^3$H) a solution of 4′-hydroxyphenylpyruvate dioxygenase (20 U) in 42 ml of the same buffer was added, and the mixture was incubated for 45 min at 25°C. The dioxygenase solution also contained 3.9 ml of a solution of dichlorophenolindophenol (3.7 μmol freshly reduced with 16.6 μmol GSH) in sodium phosphate buffer (0.02 M, pH 6.2). The reaction mixture (131 ml in total) was then cooled to 4°C, transferred into a membrane tubing, and dialyzed against distilled water (400 ml) for 18 h in the dark. (2*R*)-2′,5′-Dihydroxyphenyl[2-$^2H_1$, $^3$H]acetic acid was obtained by continuous extraction of the outside dialysate by ether. Yield: 21.3 mg (126.7 μmol, 73%) yellowish crystals with a total radioactivity of 2.02 × $10^6$ dpm [$^3$H] (0.91 μCi). The chromatographically pure substance ($R_f$ = 0.67; silica gel plate; *n*-butanol/water/formic acid, 70/25/5) was stored at −10°C under a nitrogen atmosphere.

4′-Hydroxyphenyl[3-$^{14}$C]pyruvate (0.33 μmol, 36.94 × $10^6$ dpm $^{14}$C) and (3*RS*)-4′-hydroxyphenyl[3-$^3$H]pyruvate (40.2 mg, 198.9 μmol, 1.59 × $10^9$ dpm $^3$H) were incubated in deuterated sodium phosphate buffer (97 ml, 200 mM, p$^2$H 6.2) with tautomerase (20 U, 1–2 U/mg) for 90 min at 25°C. The $^3$H/$^{14}$C ratio of the solution (both $^3$H and $^{14}$C in dpm) was 43. [Since no significant solvent isotope effect on the rate of the tautomerase reaction was observed virtually, all 3-$H_{Re}$ atoms should have been exchanged for deuterium during the first 10 min. (3*R*)-tritiated molecules competed then with the bulk of the (3*R*)-deuterated molecules. Although the discrimination of the former against the latter was not measured, the deuterium isotope effect on the overall reaction rate ($k_H/k_{2_H}$) was determined to be 8–9. For this reason we assume that most of the tritium from the (3*R*) tritiated molecules was washed out into the solvent during the reaction time applied. This is also substantiated by the subsequent stereochemical analysis. On the other hand, control experiments showed that no exchange of aromatic hydrogen atoms occurred

under the conditions of the enzymic experiment.] The reaction mixture was immediately treated with 4′-hydroxyphenylpyruvate dioxygenase (20 U) containing 4.2 ml of the same reduced dichlorophenol solution described in the previous section. The dioxygenase was dissolved in deuterated sodium phosphate buffer (40 ml, 200 mM, $p^2H$ 6.2), the total volume of the reaction mixture was 141 ml. After incubation (45 min, 25°C) the mixture was cooled to 4°C, transferred into a membrane tubing, and dialyzed against distilled water (400 ml, 18 h, 4°C) in the dark. Extraction of the outside phase as described for the other enantiomer yielded 22.9 mg (136 μmol, 68%) yellowish crystals (mp. 148°C; $49.69 \times 10^6$ dpm $^3H$, $2.46 \times 10^6$ dpm $^{14}C$; $^3H/^{14}C = 20.19$). The chromatographically pure compound was stored at −10°C under nitrogen.

(2*RS*)-2′,5′-Dihydroxyphenyl[2-$^3$H]acetic acid was prepared from (3*RS*)-4′-hydroxyphenyl[3-$^3$H]pyruvate (39.9 mg, 197.4 μmol, $23 \times 10^7$ dpm $^3H$) by oxidation in the presence of 4′-hydroxyphenylpyruvate dioxygenase (20 U). The incubation time was 60 min; the total volume of the reaction mixture was 65 ml; all other conditions were identical to those described for the previous oxidations. Yield: 23.5 mg (139.8 μmol, 71%) crystalline product ($10.74 \times 10^6$ dpm $^3H$).

*Conversion of the Labeled Homogentisates with Isolated Intact Chloroplasts from R. sativus L. cv. saxa*

The leaves of 12- to 14-day-old *Raphanus* seedlings were used to prepare the intact chloroplasts according to the method of Nakatani and Barber (*6*). The chloroplasts from 5 kg of leaves were suspended in 200 ml isolation medium (*6*) containing sodium bicarbonate (2 mM), dithiothreitol (10 mM), ATP (1 mM), and (±)-mevalonate (0.1 mM). The labeled homogentisate (in 2 ml water) was then added to this suspension. After illumination (200,000 Lux, 50 W/m$^2$) for 7 h at 25°C the chloroplast pigments were extracted with acetone (500 ml); to the dried extract a mixture of low-boiling petroleum ether and diethylether (4 : 1) was added. The pigments were chromatographed on an aluminium oxide (acidic, activity Grade III) column (3 × 8 cm) in the latter solvent system. The eluate contained in addition to some β-carotin, plastoquinone 9, α-tocoquinone, α-tocopherol, and phylloquinone (*7*). Aliquots of the product were decolorized by uv light before scintillation counting (Table 1).

*Reductive Acetylation of the Prenylquinone Mixtures* (**8**)

In an exploratory experiment α-tocoquinone (64 mg, 143 μmol) in acetanhydride (5 ml) was treated under nitrogen with zinc dust (100 mg) and triethylamine (2–3 drops). After stirring (1 h) at 25°C the mixture was cooled in an ice bath and hydrolyzed by careful addition of water. The remaining zinc was removed by filtration and the resulting clear solution was extracted with chloroform (3 × 30 ml). After washing (saturated aqueous NaCl) and drying ($Na_2SO_4$) the chloroform was removed by evaporation. Yield: 69 mg (130 μmol, 91%) 1,4-diacetyltocohydroquinone (mp 64°C). $R_f = 0.65$ (silica gel plate; ethanol/hexane/toluene, 2/2/1); $^1H$ NMR ($CDCl_3$/TMS) δ = 0.80–0.90 (m, 15 H), 1.00–1.69 (m, 25 H), 2.03 (s, 3 H), 2.05 (s, 3 H), 2.09 (s, 3 H), 2.35 (s, 6 H).

TABLE 1

INCORPORATION OF LABELED HOMOGENTISATES INTO PRENYLQUINONES IN RADISH CHLOROPLASTS

| Experiment no. | Label and absolute configuration of homogentisate | Total radioactivity (dpm) calculated from the value found in: Homogentisate before feeding | The prenylquinone fraction isolated | Incorporation (%) |
|---|---|---|---|---|
| 1 | (*RS*)-[2-$^3$H] | $10.74 \times 10^6$ [$^3$H] | $0.556 \times 10^6$ [$^3$H] | 5.18 |
| 2 | (2*R*)-[2-$^2$H$_1$, $^3$H] | $2.02 \times 10^6$ [$^3$H] | $0.224 \times 10^6$ [$^3$H] | 11.1 |
| 3 | (2*S*)-[2-$^2$H$_1$, $^3$H, $^{14}$C] | $4.96 \times 10^7$ [$^3$H] | $0.262 \times 10^6$ [$^3$H] | 0.53 |
| | | $2.46 \times 10^6$ [$^{14}$C] | $1.34 \times 10^4$ [$^{14}$C] | 0.55 |
| | | $^3$H/$^{14}$C = 20.20 | $^3$H/$^{14}$C = 19.55 | |

The prenylquinone mixtures from Experiments 1–3 were processed in the same way. In each case crystalline products (80–100 mg) were obtained. The results from the three experiments are summarized in Table 1.

*Kuhn–Roth Oxidation of the Acetylated Prenylhydroquinone Mixtures*

The acetylated prenylhydroquinone mixtures were stirred with a solution of chrom(VI)oxide (10 g) in water (25 ml) for 24 h at 25°C (*9*). Phosphoric acid (7.5 ml, 85%) was then added and the mixture was submitted to steam distillation. The distillates (ca. 250 ml) were adjusted with 0.1 M NaOH to pH 7.8–8.0 and then evaporated to dryness. An aliquot of the products was used to prepare crystalline phenacylacetate (mp 49.5°C) to determine the exact radioactivity. The results from the Kuhn–Roth degradations are summarized in Table 2.

*Analysis of the Radioactive Acetates for Chirality*

The radioactive sodium acetate samples obtained after Kuhn–Roth degradation were converted into acetyl-CoA by the combined action of acetate kinase and

TABLE 2

RESULTS FROM THE KUHN–ROTH DEGRADATION OF THE LABELED DIACETYLPRENYLHYDROQUINONES

| Experiment no. | Total radioactivity (dpm) calculated from the value found in: Diacetylprenyl-hydroquinones | Acetate solution | Phenacyl acetate |
|---|---|---|---|
| 1 | $4.79 \times 10^5$ [$^3$H] | $2.87 \times 10^5$ [$^3$H] | $2.23 \times 10^5$ [$^3$H] |
| 2 | $1.97 \times 10^5$ [$^3$H] | $1.23 \times 10^5$ [$^3$H] | $9.18 \times 10^4$ [$^3$H] |
| 3 | $2.23 \times 10^5$ [$^3$H] | $1.34 \times 10^5$ [$^3$H] | $1.01 \times 10^5$ [$^3$H] |
| | $1.15 \times 10^4$ [$^{14}$C] | $1.11 \times 10^4$ [$^{14}$C] | $8.57 \times 10^3$ [$^{14}$C] |
| | $^3$H/$^{14}$C = 19.39 | $^3$H/$^{14}$C = 12.07 | $^3$H/$^{14}$C = 11.78 |

TABLE 3

ANALYSIS OF THE RADIOACTIVE ACETATES FOR CHIRALITY

| Experiment no. | Amount of L-malate (mg) | Specific radioactivity of malate (dpm/mg) | | [$^3$H] retention (%) |
|---|---|---|---|---|
| | | Before | After fumarase treatment | |
| 1 | 77 | 606.6 [$^3$H] | 309.6 [$^3$H] | 51 |
| 2 | 68 | 146.6 [$^3$H] | 49.9 [$^3$H] | 34 |
| 3 | 60 | 142 [$^3$H] | 104.5 [$^3$H] | |
| | | 11.7 [$^{14}$C] | 12.3 [$^{14}$C] | 68.5 |
| | | $^3$H/$^{14}$C = 12.2 | $^3$H/$^{14}$C = 8.36 | |

transphosphorylase (*10, 11*). The preparations were then analyzed by the method of Cornforth *et al.* (*10, 11*) and Lüthy *et al.* (*12*). The results are summarized in Table 3. All malic acid samples were also submitted to a quantitative analysis by malate dehydrogenase (*13*) and were found to be 96.5–98% pure.

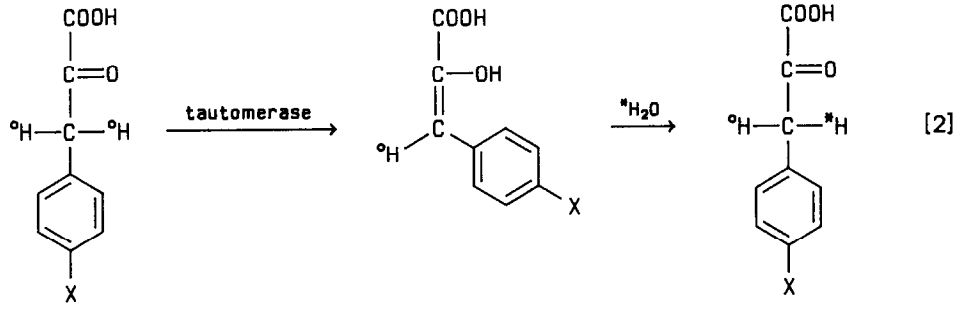
## RESULTS AND DISCUSSION

4′-Hydroxyphenylpyruvate samples were labeled in position 3 with deuterium or tritium by incubation in the corresponding medium under slightly alkaline conditions. A high degree of labeling (>95% [$^2$H] and >2 Ci [$^3$H] per mol) was aspired to and achieved by using highly labeled solvents and/or repeating the exchange experiment with fresh solvent. In the case of the deuterated sample $^1$H NMR examination showed that about one-third of the signal system at 7.1–7.4 ppm was lacking as compared with the higher-field multiplet in the aromatic region (6.7–7.0 ppm). Furthermore, $^1$H–$^2$H couplings in the latter indicated deuterium incorporation into the vicinal position. If the multiplet at lower field arises from the 3′-protons then basic treatment of 4′-hydroxyphenylpyruvate also led, besides complete exchange in position 3, to a partial exchange in position 3′ of the aromatic ring. 4′-Hydroxyphenyl[3-$^{14}$C]pyruvate was also prepared by enzymatic oxidation of [3-$^{14}$C]tyrosine.

To elucidate the steric course of the decarboxylation by which the acetic acid side chain of homogentisate is converted into a methyl group of $\alpha$-tocopherol **2** and plastohydroquinone **3** (see Eq. [1]), a stereospecific labeling of the methylene group was, however, required. For convenience the two heavy hydrogen isotopes were introduced first, while the addition of protium was left for the biological decarboxylation, which thus generated a chiral methyl group.

For the introduction of the second heavy isotope into the methylene group of 4′-hydroxyphenylpyruvate the stereospecificity of a commercially available enzyme, phenylpyruvate tautomerase, was exploited. This enzyme catalyzes the reversible enolization of phenylpyruvates by stereospecific abstraction of the $H_{Re}$ atom from

the methylene group [Eq. 2] (*3*):




[2]

*4* °H = D, *H = T
*5* °H = T, *H = D

Thus, tautomerase-catalyzed exchange with 4′-hydroxyphenyl[3-$^2H_2$]pyruvate in tritiated water yielded (3*R*)-4′-hydroxyphenyl[3-$^2H_1$, $^3H$]pyruvate (**4**), and a similar procedure with (3*RS*)-4′-hydroxyphenyl[3-$^3H$]pyruvate in deuterium oxide led to the (3*S*)-enantiomer (**5**). The latter was additionally labeled with a small amount of 4′-hydroxyphenyl[3-$^{14}C$]pyruvate. To avoid nonstereospecific spontaneous exchange the freshly prepared, doubly labeled 4′-hydroxyphenylpyruvates were not isolated, but were converted *in situ* on partially purified 4′-hydroxyphenylpyruvate dioxygenase into (2*R*)- and (2*S*)-2′,5′-dihydroxyphenyl[2-$^2H_1$, $^3H$]acetates (homogentisates), the (2*S*)-enantiomer still carrying the expected amount of $^{14}C$ in position 2. (2*RS*)-[2-$^3H$]Homogentisate was also prepared in the same way. The absolute configuration of the two chirally labeled homogentisates follows from the known stereospecificity of the dioxygenase reaction. Leinberger *et al.* (*4*) showed recently that the configuration of the chirally labeled methylene group is retained during migration of the side chain.

During isolation of the homogentisates special care was taken for the removal of traces of iron porphyrins originating from hemoglobin, a contaminant in the dioxygenase preparation. Traces of iron complexes, together with light and oxygen, caused rapid degradation of homogentisates. The separation of iron-containing protein from the labeled homogentisates was achieved by dialysis.

The incorporation of [2-$^{14}C$]homogentisate into the prenylquinones was studied in a number of preliminary experiments, in which whole seedlings of barley and radish, as well as germinating radish seeds, were used as biological catalysts. The best results were achieved, however, with intact chloroplasts isolated from radish seedlings (*R. sativus*). In the following, samples of (2*RS*)-[2-$^3H$]-, (2*R*)-[2-$^2H_1$, $^3H$]-, and (2*S*)-[2-$^2H_1$, $^3H$, $^{14}C$]homogentisate (Experiments 1–3) were incubated with chloroplasts under illumination and the prenylquinones were isolated by chromatography. It has been shown previously (*7*) that this fraction of the radish chloroplasts contains 28.5% plastoquinone-9, 21.4% plastohydroquinone-9, 40.6% α-tocopherol, 6.8% α-tocoquinone, and 2.7% phylloquinone. The incorporation of radioactivity from the homogentisates into the prenylquinone fraction varied be-

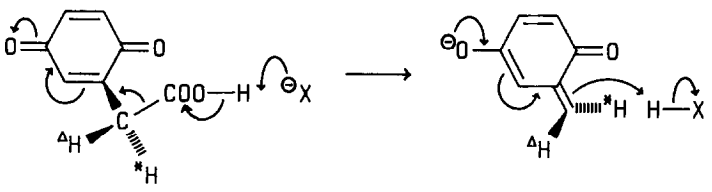


FIGURE 1

tween 0.5 and 11.8%. The results are summarized in Table 1. It is noteworthy that in Experiment 3, in which the lowest incorporation was obtained, the $^{3}H/^{14}C$ ratio remained practically constant.

The radiolabeled prenylquinone fractions were then reductively acetylated, thus converting all quinones and hydroquinones into the corresponding diacetylhydroquinones. This step was understood as a precaution, since in the quinones the *ortho*-methyl groups could lose isotopic hydrogens by reversible enolization.

The diacetylhydroquinone mixtures from Experiments 1–3 were submitted to a mild variant (*9*) of Kuhn–Roth degradation which yielded radioactive acetates. The results are summarized in Table 2. The yield of tritium radioactivity was about 60%. In Experiment 3 the $^{3}H/^{14}C$ ratio decreased from about 19 to 12, which indicates that one-third of the tritium was lost in the process. This tritium was most probably in the aromatic position of plastohydroquinone, which accounts for about half of the hydroquinone mixture. The observed base-catalyzed incorporation of deuterium into the 3′ position of the 4′-hydroxyphenylpyruvate precursor makes such an explanation plausible. If the tritium loss had occurred by reversible enolization then a greatly diminished stereochemical purity of the acetates should be expected. The stereochemical analysis of the acetates (*10–12*), however, showed the anticipated purity (Table 3).

The tritium retentions found in the malate samples after fumarase treatment indicate that in Experiment 2 about 70% excess of (*S*)-[$^{2}H_1$, $^{3}H$]acetate was produced, whereas in Experiment 3 a similar excess of (*R*)-[$^{2}H_1$, $^{3}H$]acetate was generated. The enzymatic decarboxylation thus occurred with retention of configuration, in agreement with other biological decarboxylations investigated so far (*9, 14–16*).

As shown by Leinberger *et al.* (*4*) the stereochemical purity of the chirally labeled homogentisates obtained by the same method lies between 75 and 80%. Thus only little stereochemical purity was lost during the biosynthesis of prenylquinones and/or their Kuhn–Roth degradations.

We are attempting to postulate that the decarboxylation takes place in a quinone intermediate in which an appropriate stereoelectronic set-up facilitates charge delocalization (see Fig. 1). The same basic group on the enzyme which deprotonates the carboxyl group could serve as a proton donor for the intermediate enolate. Such an array would ensure that the proton is added from the same side from which the carboxyl group has been removed.

## ACKNOWLEDGMENTS

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## REFERENCES

1. WHISTANCE, G. R., AND THRELFALL, D. R. (1970) *Biochem. J.* **117,** 593.
2. SOLL, J., AND SCHULTZ, G. (1980) *Phytochemistry* **19,** 215.
3. RÉTEY, J., BARTL., K., RIPP, E., AND HULL, W. E. (1977) *Eur. J. Biochem.* **72,** 251.
4. LEINBERGER, R., HULL, W. E., SIMON, H., AND RÉTEY, J. (1981) *Eur. J. Biochem.* **117,** 311.
5. DIXON, G. H., AND KORNBERG, H. L. (1962) *in* Methods in Enzymology (Colowick, S. P., and Kaplan, N. O., eds.), Vol. 5, p. 633, Academic Press, New York.
6. NAKATANI, H. Y., AND BARBER, J. (1977) *Biochim. Biophys. Acta* **461,** 510.
7. GRUMBACH, K. H. (1984) *Physiol. Plant.* **60,** 180.
8. RICHTER, R., AND ULRICH, H. (1977) *Houben-Weyl* **7,** 652.
9. BATTERSBY, A. R., DEUTSCHER, K. R., AND MARTINONI, B. (1983) *J. Chem. Soc. Chem. Commun.*, 698.
10. CORNFORTH, J. W., REDMOND, J. W., EGGERER, H., BUCKEL, W., AND GUTSCHOW, C. (1969) *Nature (London)* **221,** 1212.
11. CORNFORTH, J. W., REDMOND, J. W., EGGERER, H., BUCKEL, W., AND GUTSCHOW, C. (1970) *Eur. J. Biochem.* **14,** 1.
12. LÜTHY, J., RÉTEY, J., AND ARIGONI, D. (1969) *Nature (London)* **212,** 1213.
13. HOHORST, H. J. (1962) *in* Methoden der enzymatischen Analyse (Bergmeyer, H. U., ed.), Vol. 2, p. 1544, Verlag Chemie, Weinheim.
14. BARNARD, G. F., AND AKHTAR, M. (1975) *J. Chem. Soc. Chem. Commun.*, 499.
15. LIENHARD, G. E., AND ROSE, I. A. (1964) *Biochemistry* **3,** 185.
16. CHEN, R. F., AND PLAUT, G. W. E. (1963) *Biochemistry* **2,** 752.