Mechanisms of Enzymatic and Acid-Catalyzed Decarboxylations of Prephenate†

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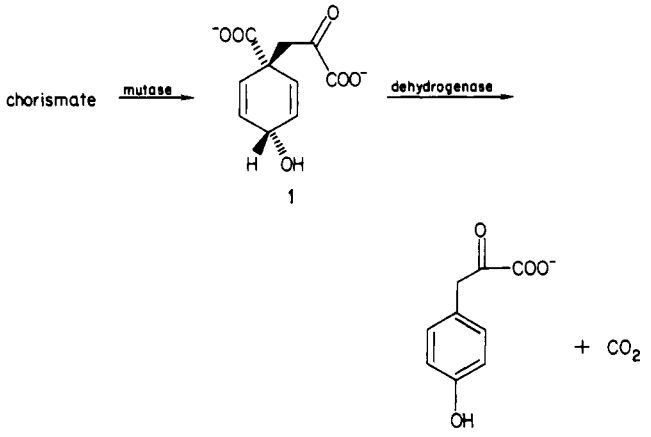
ABSTRACT: The prephenate dehydrogenase activity of the bifunctional enzyme chorismate mutase–prephenate dehydrogenase from *Escherichia coli* catalyzes the oxidative decarboxylation of both prephenate and deoxoprephenate, which lacks the keto group in the side chain (V 78% and V/K 18% those of prephenate). Hydride transfer is to the B side of NAD, and the acetylpyridine and pyridinecarboxaldehyde analogues of NAD have V/K values 40 and 9% and V values 107 and 13% those of NAD. Since the ^{13}C isotope effect on the decarboxylation is 1.0103 with deuterated and 1.0033 with unlabeled deoxoprephenate (the deuterium isotope effect on V/K is 2.34), the mechanism is concerted, and if CO_2 has no reverse commitment, the intrinsic ^{13}C and deuterium isotope effects are 1.0155 (corresponding to a very early transition state for C–C bond cleavage) and 7.3, and the forward commitment is 3.7. With deoxodihydroprephenate (lacking one double bond in the ring), oxidation occurs without decarboxylation, and one enantiomer has a V/K value 23-fold higher than the other (deuterium isotope effects are 3.6 and 4.1 for fast and slow isomers; V for the fast isomer is 5% and V/K 0.7% those of prephenate). The fully saturated analogue of deoxoprephenate is a very slow substrate (V 0.07% and V/K $\sim 10^{-5}$% those of prephenate). pH profiles show a group with pK = 8.3 that must be protonated for substrate binding and a catalytic group with pK = 6.5 that is a cationic acid (likely histidine). This group facilitates hydride transfer by beginning to accept the proton from the 4-hydroxyl group of prephenate prior to the beginning of C–C cleavage (or fully accepting it in the oxidation of the analogues with only one double bond or none in the ring). In contrast with the enzymatic reaction, the acid-catalyzed decarboxylation of prephenate and deoxoprephenate ($t_{1/2}$ of 3.7 min at low pH) is a stepwise reaction with a carbonium ion intermediate, since ^{18}O is incorporated into substrate and its epi isomer during reaction in $H_2{}^{18}O$. pH profiles show that the hydroxyl group must be protonated and the carboxyl (pK ~ 4.2) ionized for carbonium ion formation. The carbonium ion formed from prephenate decarboxylates 1.75 times faster than it reacts with water (giving 1.8 times as much prephenate as epi isomer). The observed ^{13}C isotope effect of 1.0082 thus corresponds to an intrinsic isotope effect of 1.023, indicating an early transition state for the decarboxylation step. *epi*-Prephenate is at least 20 times more stable to acid than prephenate because it exists largely as an internal hemiketal. By contrast, the isomers of deoxoprephenate decarboxylate in acid at equal rates, and the carbonium ion intermediate decarboxylates 2.6 times faster than it reacts with water (giving equal amounts of deoxoprephenate and its epi isomer). Prephenate analogues with one or no double bonds in the ring are acid stable, as are the ketones to which they are oxidized by prephenate dehydrogenase.

Chorismate mutase–prephenate dehydrogenase [chlorismate pyruvatemutase (EC 5.4.99.5)–prephenate:NAD oxidoreductase (decarboxylating) (EC 1.3.1.12)] is a bifunctional enzyme that catalyzes both the rearrangement of chlorismate to prephenate (**1**) and the oxidative decarboxylation of prephenate to (4-hydroxyphenyl)pyruvate. The work of Christopherson et al. (1983) suggests that the sites responsible for the mutase and dehydrogenase activities are distinct but very close to each other and possibly overlapping. Each reaction is irreversible, however, and the requirement for NAD in the prephenate dehydrogenase reaction allows the two activities to be studied independently. For convenience, we will refer to this enzyme as prephenate dehydrogenase.



The kinetic mechanism of the dehydrogenase reaction has been investigated by steady-state kinetic techniques, and the data are consistent with a random addition of NAD and prephenate (Sampathkumar & Morrison, 1982). Two equally feasible chemical mechanisms for the oxidative decarboxylation of prephenate can be postulated. Stepwise mechanisms, in which hydride transfer to yield a carbonyl compound precedes decarboxylation, have been demonstrated for the oxidative decarboxylations catalyzed by malic enzyme, 6-phosphogluconate dehydrogenase, and isocitrate dehydrogenase (Hermes et al., 1982; Rendina et al., 1984; Grissom & Cleland, 1983). The corresponding reaction with prephenate would yield a vinylogous analogue of a β-keto acid (Scheme Ia). One could also imagine a concerted mechanism that, because of the exothermicity of aromatization, might be the preferred (lower energy) pathway (Scheme Ib). We have resolved this question in favor of the concerted mechanism by applying the technique of Hermes et al. (1982) in which the ^{13}C kinetic isotope effect on decarboxylation is determined with both deuterated and undeuterated substrates.

We have tested the substrate specificity of prephenate de-

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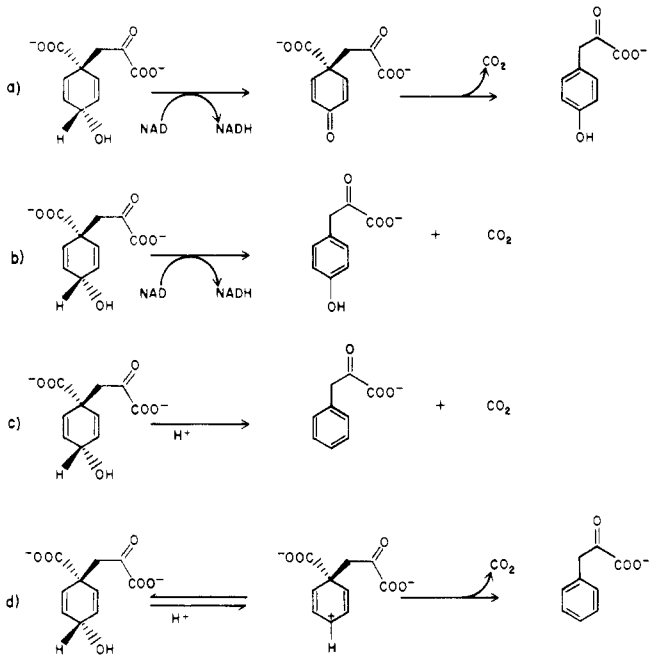
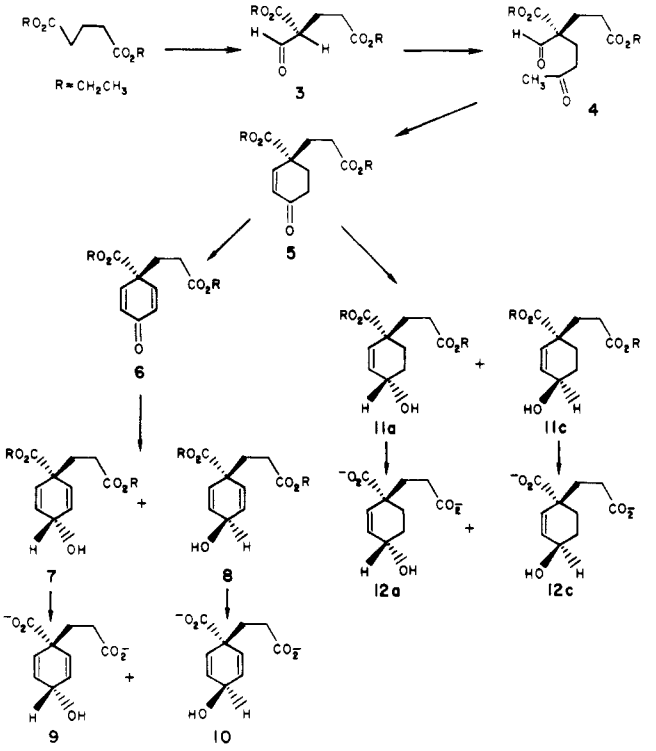
Scheme I



Scheme II



hydrogenase by studying substrate analogues in which the keto group has been removed from the side chain, which has little effect, and ones in which one or both double bonds have been removed from the ring. The latter change permits oxidation but prevents decarboxylation. We have also established the stereochemistry for hydride transfer to NAD by proton NMR methods.

For comparison with the enzymic decarboxylation, the acid-catalyzed decarboxylation of prephenate to phenylpyruvate was studied (Scheme Ic). The same two mechanisms can again be postulated: a concerted elimination of the elements of CO_2 and OH as in Scheme Ic and a stepwise mechanism proceding through a resonance-stabilized carbonium ion intermediate (Scheme Id). Partial decarboxylation of prephenate in $H_2{}^{18}O$ and examination of the residual starting material for ^{18}O incorporation were used to show that, in contrast with the enzymatic reaction, the acid-catalyzed decarboxylation has a stepwise mechanism.

Experimental Procedures

Materials. Borane-d_3 in tetrahydrofuran (1 M) was from Alfa. $H_2{}^{18}O$ (95.7 atom % ^{18}O) was from the Mound Facility of the Monsanto Research Corp. Ethanol-d_6 (99 atom % D) was from Merck. D_2O (100.00 atom % D and low in paramagnetic impurities) was from Aldrich. Chorismate mutase–prephenate dehydrogenase was isolated from *Escherichia coli* JFM 30 as described by Sampathkumar & Morrison (1982) and purified to a specific activity of 68.4 units/mg. Rabbit muscle lactate dehydrogenase, *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase, bovine glutamate dehydrogenase, 3-acetylpyridine–NAD, 3-pyridinecarboxaldehyde–NAD, sodium pyruvate, Hepes,[1] and glucose 6-phosphate were from Sigma. Crystalline lithium NAD was from Boehringer-Mannheim. Silica gel 60 (70–230 mesh) for column chromatography and silica gel 60 thin-layer plates (with F-254 fluorescent indicator and 0.25 mm layer thickness) were from E. Merck, Darmstadt, West Germany. *Aerobacter aerogenes* 62-1 was used to produce chorismic acid, which was isolated and recrystallized by the method of Gibson (1968). Sodium prephenate was prepared from chorismate by the action of chorismate mutase (Dudzinski & Morrison, 1976). The barium salt of prephenate (**1**) from Sigma contained 76% prephenate and ~20% *epi*-prephenate (**2**), presumably as the result of exposure to low pH during isolation.

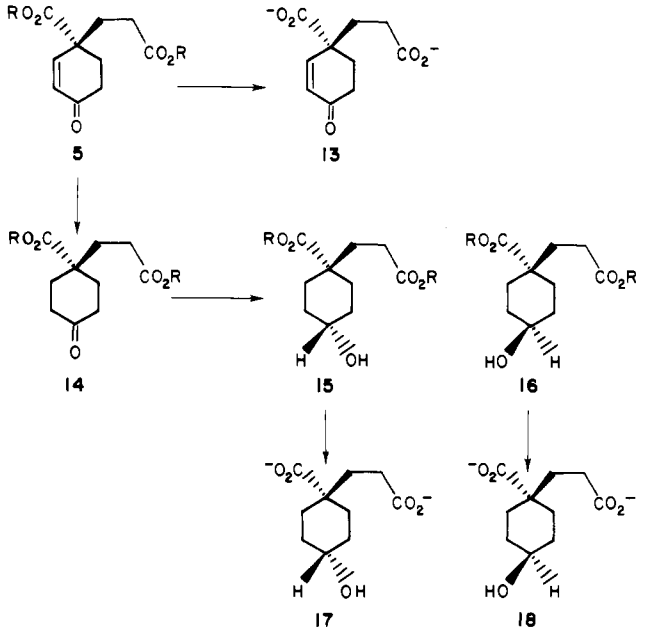
NAD deuterated at the 4-position of the nicotinamide ring (NAD-*4-d*) was synthesized by reduction of NAD with ethanol-d_6, liver alcohol dehydrogenase, and aldehyde dehydrogenase to yield A-side NADD as described by Viola et al. (1979), followed by oxidation by the method of Cook et al. (1980) using the B-side enzyme glutamate dehydrogenase. The enzymes were removed by Amicon PM-10 ultrafiltration, and the filtrate was lyophilized, dissolved in D_2O (99.8 atom % D), lyophilized, and redissolved in D_2O (100.00 atom % D). Integration of the 270-MHz proton NMR spectrum indicated that 95% of the NAD was deuterated at the 4-position. This stock solution of NAD-*4-d* was used in the proton NMR experiments to determine the side specificity of prephenate dehydrogenase.

Synthetic Methodology. Silica gel 60 (F-254) thin-layer chromatography (10 × 2.5 cm plates) was typically used to monitor reactions. Elution with solvent systems $CHCl_3$/EtOH (9:1) or benzene/EtOAC (4:1) provided analytical separation of all compounds. Visualization was by (a) fluorescence quenching with a UV lamp, (b) $NH_2OH/Fe(NO_3)_3$ spray for esters, or (c) a 2,4-dinitrophenylhydrazine spray for aldehydes and ketones. "Dry" tetrahydrofuran was from Aldrich (water content less than 0.006%) packaged under N_2 in Sure/Seal bottles. Syringes and reaction flasks were dried at least 10 h in an oven at 110 °C and cooled with a dry N_2 flush. Manipulations of moisture-sensitive reagents were carried out under a N_2 atmosphere as detailed by Kramer et al. (1975).

Synthesis of Prephenate Analogues. These syntheses are shown in Schemes II and III. Compounds **3–5** are racemic mixtures; one enantiomer is shown in Scheme II. The reduction of **5** yields two pairs of enantiomers; one isomer from each pair is shown (**11a** and **11c**). Compounds **9**, **10**, **12a** (and

[1] Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonate; Mes, 2-(*N*-morpholino)ethanesulfonate; Ches, 2-(*N*-cyclohexylamino)ethanesulfonate; EDTA, ethylenediaminetetraacetic acid.

Scheme III



its enantiomer), and **12c** (and its enantiomer) are given the trivial names deoxoprephenate, *epi*-deoxoprephenate, deoxodihydroprephenate, and *epi*-deoxodihydroprephenate, respectively.

Diethyl 2-Formylglutarate (**3**). To an ice-cold suspension of 4.4 g (0.183 mol) of NaH in 70 mL of diethyl ether containing 1 drop of absolute ethanol was added a solution of 33.9 g (0.180 mol) of diethyl glutarate and 16.0 g (0.22 mol) of ethyl formate. The reaction mixture was stirred for 5 h at 10 °C and then overnight at room temperature. The white/gray solid was washed from the reaction flask with 500 mL of diethyl ether, and after being cooled to −5 °C, 100 mL of water was carefully added to the stirred suspension. The brownish aqueous layer was separated, extracted with 200 mL of diethyl ether, cooled on an ice bath, and neutralized to pH 6.0 with 2 N H_2SO_4. The acidified aqueous phase was then twice extracted with 300 mL of diethyl ether. The organic phase was dried ($MgSO_4$) and concentrated under reduced pressure to yield 32.0 g (84.6%) of **3** (colorless oil). Compound **3** exists as a mixture of two isomeric enols rather than as the aldehyde: proton NMR ($CDCl_3$) δ 1.32 (dt, 6 H, J = 6.6 Hz, CH_2CH_3), 2.43 (dd, 4 H, J = 3.3 Hz, CH_2CH_2), 4.13 (q, 2 H, J = 6.6 Hz, CH_2CH_3), 4.27 (q, 2 H, J = 6.6 Hz, CH_2CH_3), 7.15 (d, 1 H, HOHC=C of two isomers).

4-Formyl-4-carboxy-7-oxooctanoic Acid Diethyl Ester (**4**). To 21.3 g (98.5 mmol) of **3** in 20 mL of benzene at 0 °C was added 1.7 g (15.1 mmol) of potassium *tert*-butoxide. This mixture was stirred and cooled to 0–5 °C, and 8.0 mL (98.6 mmol) of freshly vacuum-distilled methyl vinyl ketone was added dropwise. Stirring was continued for 1 h at 0–10 °C, followed by overnight stirring at room temperature. A 100-mL aliquot of diethyl ether was then added, and the solution was extracted with 92 mL of 1 N NaOH at 4 °C. The aqueous phase was again extracted with 100 mL of diethyl ether, and the organic phases were combined, dried ($MgSO_4$), and concentrated under reduced pressure to yield 12.7 g (45%) of the aldehyde **4**: clear liquid; proton NMR ($CDCl_3$) δ 1.32 (dt, 6 H, J = 6.6 Hz, CH_2CH_3), 2.18 (s, 3 H, CH_3), 2.40 (m, 8 H, CH_2), 4.18 (q, 2 H, J = 6.6 Hz, CH_2CH_3), 4.27 (q, 2 H, J = 6.6 Hz, CH_2CH_3), 9.72 (s, 1 H, CHO).

1-Carboxy-4-oxo-2-cyclohexene-1-propanoic Acid Diethyl Ester (**5**). A total of 12.7 g (44.4 mmol) of **4** was added to a solution of 0.6 mL of piperidine and 1.3 mL of acetic acid in 30 mL of benzene. Refluxing in a Dean–Stark apparatus yield 0.8 mL of water (95% of theory) in 3.5 h. After this was cooled, 100 mL of diethyl ether was added, and the solution was cooled on ice. The remainder of the workup was performed at 4 °C (cold room). A 20-mL aliquot of cold 1.0 N H_2SO_4 was added, and the phases were quickly separated. The ether layer was washed successively with 30 mL of cold water, 20 mL of cold 1.0 N NaOH, and 50 mL of cold water. The ether phase was dried at room temperature ($MgSO_4$) and concentrated under reduced pressure to yield 8.7 g (73%) of the enone **5**: UV λ_{max} (MeOH) 222 nm; proton NMR ($CDCl_3$) δ 1.28 (dt, 6 H, J = 6.6 Hz, CH_2CH_3), 2–2.5 (m, 8 H, CH_2), 4.13, (q, 2 H, J = 6.6 Hz, CH_2CH_3), 4.22 (q, 2 H, J = 6.6 Hz, CH_2CH_3), 6.02 (d, 1 H, J = 11.5 Hz, CH=CHC=O), 6.90 (d, 1 H, J = 11.5 Hz, CH=CHC=O).

1-Carboxy-4-oxo-2,5-cyclohexadiene-1-propanoic Acid Diethyl Ester (**6**).[2] A total of 9.3 g (34.7 mmol) of **5** was dissolved in 250 mL of *tert*-butyl alcohol containing 3.85 g (34.7 mmol) of SeO_2 and 5.0 mL of acetic acid. This mixture was refluxed under a N_2 atmosphere for 40 h, during which time the progress of the reaction was monitored by proton NMR. After NMR indicated completion of the oxidation, the dark-reaction mixture was cooled to room temperature, and the Se_8 was removed by filtration. The mixture was then concentrated to 20 mL and stirred for 2.5 h with 3.5 g of Raney nickel that had been deactivated by stirring with water and then dried with absolute methanol. The suspension was filtered and the filtrate concentrated under reduced pressure to yield a reddish oil. This residue was dissolved in 200 mL of diethyl ether, extracted with 2 × 100 mL of 10% $NaHCO_3$, washed with 2 × 60 mL of water, extracted with 50 mL of ice-cold 0.1 N NaOH, and ultimately washed with 200 mL of water. The light reddish ether layer was dried ($MgSO_4$) and concentrated to an oil. Chromatography over 350 g of silica gel 60 (4 × 40 cm column) with elution by increasing amounts of ethyl acetate in Skelly B (hexanes) (400 mL of 5/1 hexanes/EtOAc; 400 mL of 4/1; 600 mL of 3/1; 500 mL of 2.5/1) yielded 2.8 g (30%) of pure dienone **6**: UV λ_{max} (MeOH) 235 nm; proton NMR ($CDCl_3$) δ 1.25 (dt, 6 H, J = 6.6 Hz, CH_2CH_3), 2.2–2.4 (m, 4 H, CH_2CH_2), 4.12 (q, 2 H, J = 6.6 Hz, CH_2CH_3), 4.22 (q, 2 H, J = 6.6 Hz, CH_2CH_3), 6.40 (d, 2 H, J = 10.3 Hz, H-3 and H-5), 7.02 (d, 2 H, J = 10.3 Hz, H-2 and H-6). The major side product of the SeO_2 oxidation of **5** is 3-carboethoxy-4-(carboethoxyethyl)phenol, which results from an acid-catalyzed migration of the carboethoxy group (Marx et al., 1974). This compound elutes from the silica gel column later (hexane/EtOAc, 1.5/1) in a yellow band: proton NMR ($CDCl_3$) δ 1.25 (t, 6 H, J = 6.6 Hz, CH_2CH_3), 2.07 (s, 1 H, OH), 2.2–2.5 (m, 4 H, CH_2CH_2), 4.2 (q, 4 H, J = 6.6 Hz, CH_2CH_3), 6.42 (d, 1 H, J = 12 Hz), 7.12 (m, 1 H), 7.41 (d, 1 H, J = 4 Hz).

1-Carboxy-4-hydroxy-2,5-cyclohexadiene-1-propanoic Acid Diethyl Ester (**7** *and* **8**). To a solution of **6** (1.0 g, 3.8 mmol) in 20 mL of dry tetrahydrofuran was added at room temperature under N_2 15.2 mL of 0.5 M 9-borabicyclo[3.3.1]-

[2] The conversion of enone **5** to the dienone **6** was also accomplished by a series of three reactions giving an overall yield of nearly 50%. First, the enol acetate of **5** was synthesized by using isopropenyl acetate (77%). Then, 1-carboethoxy-1-(carboethoxyethyl)-5-bromo-2-cyclohexen-4-one was made from the enol acetate by using recrystallized *N*-bromosuccinimide in CCl_4 at reflux (93%). The bromide was then eliminated by reflux with lithium carbonate in dimethylformamide (67%). Though the yield of **6** was somewhat improved by using this route, the ease of purification of **6** from the side products of the SeO_2 oxidation led us to report the details of that procedure.

nonane (9-BBN) in tetrahydrofuran. [Reduction of **6** with $NaBH_4$ gives mostly 1,4-addition products (i.e., singly allylic alcohols) and some hydride addition to the ester to yield ethyl formate and the appropriate phenol; this problem in the $NaBH_4$ reduction of dienones was also noted by Danishefsky et al. (1979) with similar compounds.] After this was stirred for 2 h, the excess reducing agent was decomposed with 10 mL of methanol, followed by 10 mL of water. The mixture was then diluted with 200 mL of water and extracted with 3 × 150 mL of $CHCl_3$. The $CHCl_3$ layer was dried ($MgSO_4$) and the volatiles were evaporated in vacuo to give a residue that was chromatographed over 350 g of silica gel 60. Elution with diethyl ether provided (after discarding the early boron-containing fractions) 0.81 g (80%) of the epimeric dienols (**7** and **8**) as a slightly yellow oil: proton NMR ($CDCl_3$) δ 1.18 (dt, 6 H, J = 6.6 Hz, CH_2CH_3), 2.01 (s, 1 H, OH), 2.20 (m, 4 H, CH_2CH_2), 4.08 (dq, 4 H, CH_2CH_3), 4.4, (br, 1 H, CHOH), 6.0 (m, 4 H, CH=CH).

1-Carboxy-4-hydroxy-2,5-cyclohexadiene-4-d-1-propanoic Acid Diethyl Ester (**7-d** *and* **8-d**). The procedure for the synthesis of **7** was followed except that 9-BBN-*9-d* replaced 9-BBN: proton NMR ($CDCl_3$) δ 1.18 (dt, 6 H, J = 6.6 Hz, CH_2CH_3), 2.01 (s, 1 H, OH), 2.20 (m, 4 H, CH_2CH_2), 4.08 (dq, 4 H, CH_2CH_3), 6.0 (m, 4 H, CH=CH).

9-Borabicyclo[3.3.1]nonane-9-d (*9-BBN-9-d*). The procedure of Brown (1975) was used to synthesize 9-BBN-*9-d* from 17.0 mL (0.017 mol) of 1 M borane-d_3 in tetrahydrofuran and 2.09 mL (0.017 mol) of cyclooctadiene. It was found that crystallization of the 9-BBN-*9-d* and removal under vacuum of any unreacted BD_3–tetrahydrofuran was necessary to prevent overreduction of **6**. 9-BBN-*9-d* crystallized when half of the solvent was evaporated and the solution cooled to −13 °C. The tetrahydrofuran was carefully removed from the white solid with a dry syringe, and 4 mL of olefin-free pentane was added; the solution warmed to room temperature and then cooled to −18 °C to recrystallize the 9-BBN-*9-d*. After removal of the solvent with a dry syringe, the solid 9-BBN-*9-d* was dried under vacuum and dissolved in dry tetrahydrofuran to yield a 1.0 M solution that was used on the same day to minimize label loss through exchange (Midland & Greer, 1978).

1-Carboxy-4-hydroxy-2,5-cyclohexadiene-1-propanoic Acid Disodium Salt (**9** *and* **10**). To a solution of dienol **7** (500 mg, 1.9 mmol) in 2.5 mL of methanol was slowly added 2.17 mL of 2 N NaOH (4.34 mmol). The solution was allowed to stir at room temperature for 5 h or until the amount of **9** was unchanged by end-point assay with prephenate dehydrogenase and excess NAD. The volatiles were removed in vacuo, first with a water aspirator and then with a vacuum pump. The residue was triturated with 2 mL of cold absolute methanol to yield a white powder. Filtration and washing with diethyl ether gave 390 mg (80%) of a mixture of **9** (deoxoprephenate) and **10** (*epi*-deoxoprephenate). Enzymatic assay of several preparations indicated that the ratio of **9** to **10** was between 0.72 and 1.0. Proton NMR (D_2O) showed the following: δ 1.9 (m, 4 H, CH_2CH_2), 4.4 (m, 1 H, CHOH), 5.83 (d, J = 7 Hz), 5.95 (q, J = 10 Hz). Signals at 5.83 and 5.95 together integrated for 4 H. One set corresponds to the epi isomer (**10**) and the other to the natural configuration (**9**) (we did not determine which was which). ^{13}C NMR (D_2O) of **9** showed the following: δ 33.79, 35.72 (exocyclic methylenes), 51.74 (C-1), 62.58 (C-4), 128.11 and 133.17 (vinyl carbons), 164.4 and 164.8 (carboxyls). ^{13}C NMR for **10** showed the following: δ 34.31, 34.85, 51.64, 62.39, 127.78, 133.06, 164.4, and 164.8. [The ^{13}C NMR resonances corresponding to the enzymatically active isomer **9** (cis relationship between the ring carboxyl and the hydroxyl group) were established by depletion of this isomer from the mixture of **9** and **10** in the presence of NAD and prephenate dehydrogenase.]

1-Carboxy-4-hydroxy-2,5-cyclohexadiene-4-d-1-propanoic Acid Disodium Salt (**9-d** *and* **10-d**). The procedure for the synthesis of **9** was followed except that the deuterated dienol **7-d** was used: proton NMR (D_2O) δ 1.9 (m, 4 H, CH_2CH_2), no signal at 4.4 ppm, 5.83 (d, J = 7 Hz), 5.95 (q, J = 10 Hz). Signals at 5.83 and 5.95 together integrated for 4 H.

1-Carboxy-4-hydroxy-2-cyclohexene-1-propanoic Acid Diethyl Ester (**11**). To a solution of 1.0 g (3.7 mmol) of enone **5** in 20 mL of dry tetrahydrofuran was added 16 mL of 0.5 M 9-BBN (all operations under a static N_2 atmosphere). After this was stirred for 1.5 h at room temperature, 10 mL of methanol followed by 10 mL of water was added to decompose unreacted 9-BBN and to hydrolyze boron esters. The solution was then diluted into 100 mL of water and extracted with 4 × 10 mL of $CHCl_3$. The chloroform phase was dried ($MgSO_4$), concentrated under reduced pressure, and chromatographed over 350 g of silica gel 60 with elution by diethyl ether to yield 0.55 g (55%) of a mixture of **11a**, **11c**, and their enantiomers: proton NMR ($CDCl_3$) δ 1.24 (dt, 6 H, J = 6.6 Hz, CH_2CH_3), 1.9 (m, 4 H, ring CH_2CH_2), 2.28 (m, 4 H, exocyclic CH_2CH_2), 3.9 (br, 1 H, CHOH), 5.8 (dd, 2 H, J = 10.8 Hz, CH=CH). The two sets of diastereomers can be separated by HPLC [silica gel, elution with *n*-hexane/EtOAc (55/45), flow rate 2.5 mL/min; retention times 3.75 and 4.25 min].

1-Carboxy-4-hydroxy-2-cyclohexene-4-d-1-propanoic Acid Diethyl Ester (**11-d**). The procedure used for synthesis of **11** was followed except that reduction of **5** was achieved with 9-BBN-*9-d*: proton NMR ($CDCl_3$) δ 1.24 (dt, 6 H, J = 6.6 Hz, CH_2CH_3), 1.85 (m, 4 H, ring CH_2CH_2), 2.28 (m, 4 H, exocyclic CH_2CH_2), 5.8 (dd, 2 H, J = 10.8 Hz, CH=CH).

1-Carboxy-4-hydroxy-2-cyclohexene-1-propanoic Acid Disodium Salt (**12**). Hydrolysis of the diethyl ester **11** was achieved by the same procedure used for **7** in the synthesis of **9**. Since **11** is a mixture of four isomers, the hydrolysis yields four compounds: **12a** and its enantiomer (**12b**) are referred to as deoxoprephenate; **12c** and its enantiomer (**12d**) are *epi*-deoxoprephenate. Proton NMR (D_2O) showed the following: δ 1.4–2.2 (m, 8 H, methylene protons), 4.1 (m, 1 H, CHOH), 5.90 (d, 2 H, J = 10.0 Hz, CH=CH).

1-Carboxy-4-hydroxy-2-cyclohexene-4-d-1-propanoic Acid Disodium Salt (**12-d**). The synthesis was the same as for **12** except that **11-d** was used: proton NMR (D_2O) δ 1.4–2.2 (m, 8 H, methylene protons), 5.90 (d, 2 H, J = 10.0 Hz, CH=CH); ^{13}C NMR (D_2O) δ 28.6, 29.5, 30.2 (exocyclic methylene carbons), 34.1, 34.2, 37.0, 37.4 (ring methylene carbons, C-5 and C-6), 49.8 (C-1), 65.4 and 67.7 (C-4), 128.6, 130.3, 135.7, 136.5 (vinyl carbons, C-2 and C-3), 164.37 (propionyl carboxyl), 164.73, 164.80 (ring carboxyl).

1-Carboxy-4-oxo-2-cyclohexene-1-propanoic Acid Disodium Salt (**13**). A total of 1.0 g (3.7 mmol) of **5** was dissolved in 4.0 mL of methanol, and 4.0 mL of 2 N NaOH (4.0 mmol) was added slowly. The solution was stirred at room temperature for 3 h. Evaporation of the volatiles left a thick oil that, upon trituration with cold methanol, produced 416 mg of **13** in the form of a white powder: UV λ_{max} (water, pH 7) 239 nm (ϵ = 9700), (water, pH 1.1) 227 nm (ϵ = 11 900); proton NMR (D_2O, pD 8.0) δ 1.85–2.20 (m, 6 H, exocyclic CH_2CH_2, 2 H-6), 2.45 (dd, 2 H, CH_2C=O), 5.92 (d, 1 H, J = 10.1 Hz, C=CHC=O), 7.11 (d, 1 H, J = 10.1 Hz, CH=CHC=O); ^{13}C NMR (D_2O, pD 8.0) δ 32.2, 34.1, 35.6,

35.8 (methylene carbons), 50.6 (C-1), 127.7 (C-3), 159.1 (C-2), 181.7, 183.6 (carboxyl carbons), 205.8 (C-4 carbonyl).

1-Carboxy-4-oxocyclohexane-1-propanoic Acid Diethyl Ester (**14**). To 50 mL of 95% ethanol was added 100 mg of 10% Pd–C catalyst that had been prereduced with H_2 at 50 psi on a Parr hydrogenation apparatus for 15 min. A total of 1.0 g of **5** was added and reduction was allowed to proceed for 90 min. The catalyst was removed by filtration through a Celite pad, and the filtrate was concentrated to 800 mg (80%) of a clear oil: proton NMR ($CDCl_3$) δ 1.3 (dt, 6 H, J = 6.6 Hz, CH_2CH_3), 1.6–1.9 (m, 4 H, H-2 and H-6), 2.4 (m, 8 H, H-3, H-5, H-α, H-β), 4.2 (dq, 4 H, J = 6.6 Hz, CH_2CH_3), no vinyl resonances.

1-Carboxy-4-hydroxycyclohexane-1-propanoic Acid Diethyl Ester (**15** *and* **16**). A total of 800 mg (3.0 mmol) of **14** was dissolved in 25 mL of absolute ethanol. A 75-mg (2.0-mmol) aliquot of $NaBH_4$ was added, and the mixture was stirred at room temperature for 2 h. One drop of 2 N NaOH was added and the solution immediately diluted with 35 mL of diethyl ether followed by 35 mL of water. The ether layer was washed with 35 mL of 1 N acetic acid and 50 mL of water. After being dried ($MgSO_4$), the ether solution was concentrated to give 500 mg (63%) of the epimers **15** and **16**: proton NMR ($CDCl_3$) δ 1.3 (t, 6 H, J = 6.6 Hz, CH_2CH_3), 2.12 (s, 1 H, OH), 1.6–2.0 (m, 8 H) 2.28 (m, 4 H), 3.6 (m, 1 H, CHOH), 4.2 (q, 4 H, J = 6.6 Hz, CH_2CH_3).

1-Carboxy-4-hydroxycyclohexane-1-propanoic Acid Disodium Salt (**17** *and* **18**). Hydrolysis of the mixture of **15** and **16** was achieved by the same procedure used for preparation of **9**.

Instrumentation. UV spectra were obtained with a Cary 118 spectrophotometer. Initial velocity studies were performed by monitoring absorbance changes with either a Cary 118 or a Beckman DU monochromator equipped with a Gilford OD converter and a 10-mV recorder. Temperatures were kept at ±0.1 °C with thermospacers and circulating water bath. pH values were measured with a Radiometer 26 pH meter equipped with a combined microelectrode standardized to ±0.01 pH unit. Ultrafiltration to remove proteins was achieved with a Series 80 Amicon ultrafiltration cell and type PM-10 (nominal M_r rejection of 10 000) semipermeable membrane filter. ^{13}C NMR spectra were recorded at 50.1 MHz on a Nicolet NT-200 Fourier-transform spectrometer equipped with either a 12-mm broad band observe probe or a 5-mm fixed-tune probe. Chemical shifts are reported as δ values in ppm relative to tetramethylsilane; dioxane (δ 67.4) was used as the actual external standard. Proton NMR spectra were obtained with either the NT-200 instrument (at 200 MHz) or a Bruker WH-270 spectrometer. Chemical shifts are reported as δ values with respect to tetramethylsilane (internal standard). The isotopic composition of CO_2 samples was determined with a Nuclide Associates RMS 6-60 isotope ratio mass spectrometer equipped with a dual-inlet system. The low- and complete-conversion samples from a particular experiment were always analyzed on the same day to minimize any day to day variations in the mass spectrometer.

Nomenclature. The nomenclature used is that of Northrop (1977), in which isotope effects on kinetic or thermodynamic parameters are defined by leading superscripts. Thus, 13, 15, 18, D, or T refers to ^{13}C, ^{15}N, ^{18}O, deuterium, or tritium isotope effects, respectively. For a further discussion of nomenclature, see Cook & Cleland (1981).

Initial Velocity Studies. All kinetic studies were run in 3.0-mL total volume, 1 cm path length cuvettes. Prephenate dehydrogenase activity was measured spectrophotometrically at 25 °C by following the formation of NADH from NAD at 340 nm. Assays typically contained 100 mM Hepes, pH 8.0, 1 mM dithiothreitol, and 1 mM EDTA.

Deuterium isotope effects on initial velocities were obtained at saturating nucleotide concentration by varying the levels of deuterated and nondeuterated substrates. V/K isotope effects determined by comparing the slopes of reciprocal plots are not sensitive to the prescence of inhibitors in the deuterated or unlabeled substrates, but their accuracy is limited by the accuracy with which the relative concentrations of the stock solutions of the labeled and unlabeled substrates are known (see below for determination of substrate concentrations). The V isotope effects do not depend on substrate calibration but are sensitive to the presence of impurities in the substrates (since we were primarily interested in the V/K isotope effects, special efforts to assure purity of the prephenate analogues were not made).

The pH dependence of V and $V/K_{prephenate}$ was determined by varying the concentration of prephenate at 750 μM NAD (saturating at all pH values investigated). The 100 mM buffers used (K-Mes, pH 5.17–6.68; K-Hepes, pH 6.61–8.51; potassium pyrophosphate, pH 7.90–9.82) contained 1 mM EDTA and dithiothreitol. In pyrophosphate buffer, the V and $K_{prephenate}$ values were higher than those in Hepes at overlapping pH values, so the V values were normalized. Ches (100 mM) was very inhibitory and could not be used as the high pH buffer. The pH dependence of $V/K_{deoxoprephenate}$ was determined by varying the concentration of deoxoprephenate at saturating levels of NAD (750 μM). A three-component buffer designed to keep ionic strength constant (Ellis & Morrison, 1982) and consisting of 100 mM Mes, 51 mM *N*-ethylmorpholine, and 51 mM diethanolamine was used over the pH range 5.5–9.5. The velocities in this buffer system did not differ significantly from those in 100 mM Hepes at the same pH.

Reaction velocities were also measured in buffers containing 20% dimethyl sulfoxide at pH values from 6.0 to 7.5. The pH values in the cuvettes were determined after completion of the spectrophotometric assay (addition of dimethyl sulfoxide raised the pH of the buffers from 0.25 unit at lower pH to 0.1 unit at higher pH). For measurement of the temperature dependence of the $V/K_{prephenate}$ pH profile, the pH meter was calibrated at the temperature of the experiment with standard buffers having known temperature coefficients.

The rate of the acid-catalyzed decarboxylation of prephenate was determined by fixed-time assays in which 10-μL aliquots of the reaction mixture (initially containing 20 mM prephenate in 0.5 mL) were removed at various times and assayed for remaining prephenate by addition to 2.5 mL of a solution containing 100 mM Hepes, 1 mM each of NAD, EDTA, and dithiothreitol, and 1 unit of prephenate dehydrogenase. Three time points were checked for each pH, with the latest near one half-life. The rate of the acid-catalyzed decarboxylation of deoxoprephenate was determined by a continuous assay of the change of absorbance at 258 nm due to the formation of phenylpropionate (ϵ = 160). The time courses were plotted in semilog form, and the first-order rate constants were obtained from the slopes. pH control was by HCl (pH 1.0–3.0) or 0.1 M acetate (pH 2.6–5.5).

Determination of Substrate Concentrations. Prephenate and deoxoprephenate concentrations were determined enzymatically with prephenate dehydrogenase as noted above. NAD, 3-acetylpyridine–NAD, and 3-pyridinecarboxaldehyde–NAD concentrations were determined enzymatically with glucose-6-phosphate dehydrogenase and 20 mM glucose 6-phosphate at pH 8. Deoxodihydroprephenate consists of two

enzymatically active isomers differing in V/K values by a factor of 23. The recorder tracing of A_{340} vs. time from reaction of this substrate with 2 mM NAD in the presence of prephenate dehydrogenase was fitted to eq 5 to determine the concentration of each isomer. The concentration in the cuvette was kept below the K_m, since one must be in the V/K concentration region to obtain data that fit eq 5.

^{13}C Kinetic Isotope Effects. The ^{13}C isotope effects on V/K for prephenate or deoxoprephenate and deoxoprephenate-*d* were determined by the method of O'Leary (1980), in which the natural abundance of ^{13}C in the substrate is used as a trace label. The assay solutions consisted of 10 mM prephenate or analogue, 13 mM pyruvate (to reoxidize NADH), and 1 mM each of NAD, EDTA, and dithiothreitol in 10 mM K-Hepes, pH 7.65. The assay solution was degassed for at least 6 h with CO_2-free N_2 before the prephenate or analogue was added. After additional degassing for 1 h, lactate dehydrogenase (100 units) and sufficient prephenate dehydrogenase to give 15–25% reaction of a 20-mL sample in 2 h were added. The reactions were carefully adjusted to pH 5.9 by adding 31 μL of concentrated H_2SO_4, and the CO_2 was isolated as quickly as possible (~14 min) on a high-vacuum line.[3] The 100%-conversion samples were treated identically except that a 5.0-mL volume of the 10 mM substrate mixture was used and was allowed to incubate overnight with 3 times the level of prephenate dehydrogenase and 200 units of lactate dehydrogenase.

For determination of the ^{13}C isotope effect on the acid-catalyzed decarboxylation of prephenate, the following modifications of the above procedure were used. Four 20-mL volumes of 150 mM Mes at pH 5.15 and 3 mL of 0.2 M disodium prephenate at pH 7.5 were degassed with CO_2-free N_2 for 6 h. The degassed Mes solutions were placed in sealed reaction vessels of the type described by O'Leary (1980), except of smaller (40 mL) size, and degassed an additional 30 min. A 1.0-mL aliquot of the degassed prephenate solution was added to the low-conversion reaction vessels, and 0.30 mL was added to the other pair (100% reactions). After 20 min (time calculated from pH rate profile for 10–15% reaction), 0.1 mL of saturated KOH was added to the low-conversion reactions to bring the pH to 6.1, and the CO_2 was isolated on a high-vacuum line. Isolation took 20 min, and therefore, negligible decomposition occurred. The 100%-conversion samples were isolated similarly on the next day.

^{13}C NMR Analysis of Acid-Catalyzed Decomposition in $H_2{}^{18}O$. A total of 25 mg of sodium prephenate was dissolved in 0.5 mL of $H_2{}^{18}O$ (97.6% ^{18}O) and adjusted to pH 4.5 with 12 N HCl. The reaction was allowed to proceed (~80 min) until 85% of the prephenate had decarboxylated as monitored by assay with prephenate dehydrogenase after neutralization of an aliquot. The pH was maintained at 4.5 during the reaction by small additions of 12 N HCl (20-μL total). The pH was then adjusted to 8.0 with concentrated KOH, 0.3 mL of D_2O was added for field lock, and a broad-band proton-decoupled ^{13}C NMR spectrum was taken. Analogous experiments with deoxoprephenate were performed in which 25- and 50-mg samples of a mixture of **9** and **10** in 0.5 mL of $H_2{}^{18}O$ (96.0% ^{18}O) were allowed to decarboxylate to extents of 84 (55 min) and 67% (35 min). These reactions were run at pH 5.0 and adjusted to pH 8.5 after the indicated time, lyophilized, and dissolved in 0.5 mL of D_2O for ^{13}C NMR analysis. Integrations of the ^{16}O- and ^{18}O-containing peaks were performed by using the Nicolet NMCCAP curve-deconvolution program, which permits an analysis of a spectrum of overlapping peaks.

Proton NMR Determination of Side Specificity of Prephenate Dehydrogenase. The stereochemistry of the hydride transfer to NAD was determined by using A-face (lactate dehydrogenase) and B-face (glutamate dehydrogenase) enzymes to deduce the location of the deuterium label in the NADD produced from the reaction of NAD-*4-d* with prephenate dehydrogenase. To 3.0 mL of 50 mM phosphate, pH 7.5, containing 15 μmol of NAD-*4-d* and 18 μmol of sodium prephenate was added either 40 μmol each of α-ketoglutarate and NH_4Cl or 40 μmol of pyruvate. A total of 1.6 units of prephenate dehydrogenase was added to each of the reaction mixtures, and the absorbance at 340 nm was monitored. When no further change in A_{340} was observed, 35 units of glutamate dehydrogenase was added to the reaction containing α-ketoglutarate and NH_4Cl, and 25 units of lactate dehydrogenase was added to the mixture containing pyruvate. After oxidation of NADD was complete (monitored at 340 nm), each reaction mixture was Amicon PM-10 filtered to remove the enzymes. The NAD was purified on a column of MP-1 macroporous ion-exchange resin, with elution by 0.2 M NaCl, pH 7. The fractions containing NAD were pooled and lyophilized. After they were dissolved in D_2O, 270-MHz proton NMR spectra were obtained of the two samples.

Data Analysis. Data from initial velocity studies of enzymatic reactions were first plotted to check the linearity of reciprocal plots. A fit of each set of data was then made to the appropriate rate equation by using the Fortran programs of Cleland (1979). Data were fitted to eq 1 when prephenate

$$v = VA/(K + A) \quad (1)$$

(or analogue) was varied at a constant level of NAD and to eq 2 when both prephenate and nucleotide concentrations were

$$v = VAB/(K_{ia}K_b + K_aB + K_bA + AB) \quad (2)$$

varied. Data were fitted to eq 3 to determine deuterium isotope

$$v = VA/[K(1 + F_iE_{V/K}) + A(1 + F_iE_V)] \quad (3)$$

effects, where F_i is the fraction of deuterium label in the substrate and E_V and $E_{V/K}$ are the isotope effects minus one on V and V/K.

^{13}C kinetic isotope effects were determined from eq 4, where

$$^{13}(V/K) = \ln(1 - f)/\ln[1 - f(R_f/R_0)] \quad (4)$$

R_f is the ^{13}C to ^{12}C ratio in CO_2 at fraction of reaction f and R_0 is the mass ratio at $f = 1.0$.

For analysis of rate constants and substrate concentrations for the biphasic time courses observed with deoxodihydroprephenate, the data were fitted to eq 5, where y is NADH

$$y = -Ae^{-kt} + Bt + A \quad (5)$$

concentration, k is the V/K value, A is the concentration of the fast isomer, and B/A is the V/K value for the slow isomer. The concentrations of the fast and slow isomers were assumed to be equal since they are enantiomers and an achiral reducing agent was used in their synthesis.

[3] To isolate CO_2 from the aqueous assay mixture, the pH must be lowered below the pK of bicarbonate (6.4). At this pH, the half-times for the acid-catalyzed decarboxylation of prephenate and deoxoprephenate are 540 and 155 min, respectively. At an enzymatic conversion of 25% reaction and a collection period of 14 min at pH 5.9, ~5 and ~15% of the CO_2 collected were from the nonenzymatic, acid-catalyzed reactions of prephenate and deoxoprephenate, respectively. The magnitude of ^{13}k on the acid-catalyzed decarboxylation (1.0082) is small enough that this contamination does not introduce serious errors in the calculated ^{13}C isotope effects for the enzymatic reactions (the corrections that were calculated were less than 0.1% in the ^{13}C isotope effects).

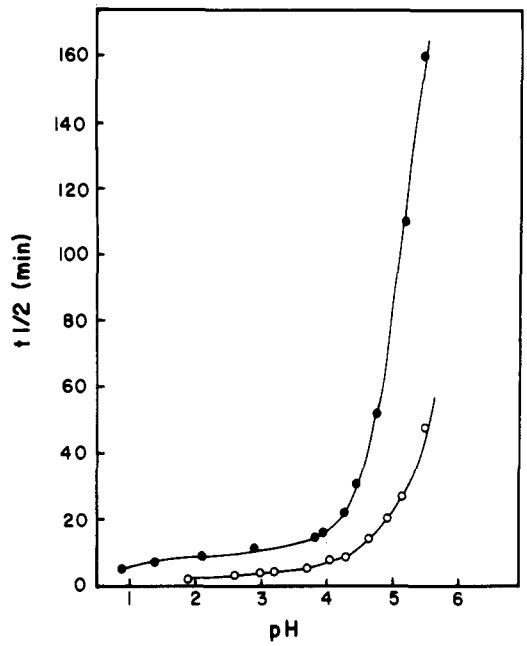


FIGURE 1: Half-life in minutes for the acid-catalyzed decarboxylation of (●) prephenate and (○) deoxoprephenate.

The pH profiles for prephenate dehydrogenase were fitted to eq 6 or 7, where y is V for eq 6 and V/K for eq 7, C is the

$$\log y = \log [C/(1 + [H^+]/K_1)] \quad (6)$$

$$\log y = \log [C/(1 + [H^+]/K_1 + K_2/[H^+])] \quad (7)$$

pH-independent value of y, and K_1 and K_2 are acid dissociation constants. The pK values determined in the temperature-dependence studies were fitted to eq 8. The half-life values

$$pK = \Delta H_{ion}/(2.303RT) \quad (8)$$

for acid-catalyzed decomposition of prephenate were fitted to eq 9, where K_1, K_2, and K_3 are acid dissociation constants of

$$\log t_{1/2} = \log \frac{(C[1 + [H^+]/K_1 + [H^+]/K_2 + [H^+]^2/(K_1K_3)])}{[H^+](1 + [H^+]/K_1)} \quad (9)$$

the pyruvyl carboxyl and of the ring carboxyl with the pyruvyl carboxyl ionized or protonated. C/K_3 is the half-time at very low pH, and C/K_2 is the value in the pH range between pK_1 and pK_2. Rate constants for the acid-catalyzed decomposition of deoxoprephenate were fitted to eq 10, where K_2 is the same

$$\log k = \log [C/(1 + K_2/[H^+])] \quad (10)$$

pK as in eq 9 and C is the low pH value of k.

Results

pH Dependence of Acid-Catalyzed Decarboxylation of Prephenate and Prephenate Analogues. Both prephenate and deoxoprephenate decompose in a firstorder fashion, and the pH variation of the half-times is shown in Figure 1. The data for prephenate were fitted to eq 9, giving values of 1.4 ± 0.5, 4.2 ± 0.1, and 3.7 ± 0.3 for pK_1, pK_2, and pK_3 and half-times of 3.7 ± 0.1 min below pK_1 and 10.6 ± 0.1 between pK_1 and pK_2. The data for deoxoprephenate were fitted to eq 10, giving 4.3 ± 0.1 as the pK and 3.7 ± 0.4 min as the limiting half-time at low pH. The rate of decarboxylation of deoxoprephenate is nearly 3 times that of prephenate in the pH range 3–5.5.

Deoxodihydroprephenate (**11**) is stable in 1 M acetic acid at pH 3.1, 25 °C, and has a half-life greater than 320 h in 1 N HCl at 25 °C, as determined by fixed-time assays with prephenate dehydrogenase.

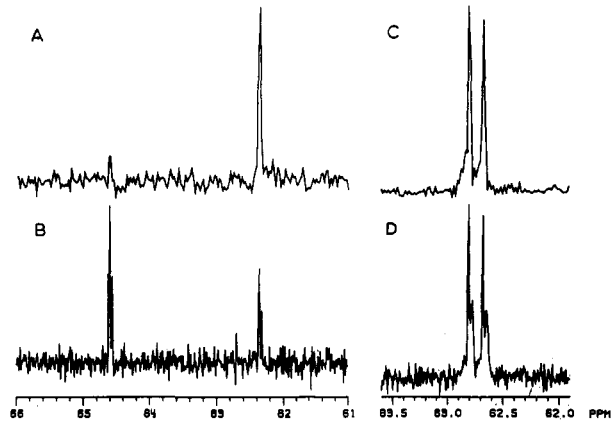


FIGURE 2: Limited-acquisition ^{13}C spectra of the C-4 carbons of prephenate (A), prephenate after 85% decarboxylation in $H_2{}^{18}O$ (B), deoxoprephenate (C), and deoxoprephenate after 67% decarboxylation in $H_2{}^{18}O$ (D). Prephenate contained a small amount of the epi isomer, while the synthetic deoxoprephenate was ~50% *epi*-deoxoprephenate. Acquisition parameters were as follows: 8.00-μs transmitter pulse (90° pulse), 8K block size, 1000-Hz sweep width (4.10-s acquisition time), and bilevel, broad-band proton decoupling.

Stability of Ketones. The UV and proton NMR spectra of **13** were unchanged after 2 h at pH 3. Even in 1 N HCl at 80 °C, decarboxylation of this vinylogous β-keto acid could not be detected. However, incubation at 78 °C in 1 M morpholine, pH 9.5, did promote decarboxylation ($t_{1/2}$ = 30 min) by formation of a cationic imine intermediate. The production of 4-oxo-2-cyclohexene-1-propanoate was verified by proton NMR and thin-layer chromatography [the monocarboxylic acid has an R_f of 0.48 on silica gel 60 with a toluene/AcOH (12/7) elution].

Attempts to produce the dicarboxylic acid corresponding to **6** by saponification of **6** in NaOH/MeOH resulted only in the formation of a phenol and ethyl carbonate. The tetrahedral intermediate formed by attack of hydroxide on the ring carboxylic ester presumably releases the ethyl ester of (*p*-hydroxyphenyl)propionate rather than ethoxide ion as the result of the exothermicity of this aromatization.

^{13}C NMR Analysis of Decarboxylation of Prephenate and Deoxoprephenate in $H_2{}^{18}O$. The limited acquisition ^{13}C NMR spectrum of C-4 of prephenate shows a single peak with a line width at half-height of 0.04 ppm at 62.4 ppm (Figure 2A). After 85% decarboxylation in $H_2{}^{18}O$ at pH 4.5, a resonance 0.037 ppm upfield of the C-4 prephenate peak appears that is due to prephenate-*4-*^{18}O (Figure 2B). The ratio of $^{18}O/^{16}O$ species on the basis of integration was 0.55. An additional doublet with a chemical shift difference appropriate for an ^{18}O perturbation appeared at 64.6 ppm. The downfield peak of this doublet corresponded to a *epi*-prephenate contaminant in the prephenate (Figure 2A), and the upfield peak is *epi*-prephenate-*4-*^{18}O formed during the reaction. The $^{18}O/^{16}O$ ratio in *epi*-prephenate was 0.87. The peak corresponding to *epi*-prephenate did not change in intensity by more than 10% over the course of the reaction (that is, it is relatively acid stable).

Analogous experiments were performed with deoxoprephenate (**9** and **10**). Figure 2C shows the ^{13}C NMR spectrum of C-4 of **9** (62.80 ppm) and **10** (62.66 ppm) before decarboxylation, and Figure 2D shows the spectrum after 67% decarboxylation in $H_2{}^{18}O$. The peaks corresponding to ^{18}O-labeled **9** and **10** are 0.034 ppm upfield and integrate for ~36% of the peaks of the ^{16}O-containing compounds.

^{13}C Kinetic Isotope Effects on Acid-Catalyzed Decarboxylation of Prephenate. This isotope effect was determined at pH 5.15 (150 mM Mes, 25 °C) by comparison of $^{13}C/^{12}C$ ratios in CO_2 from partial and complete reactions (Table I).

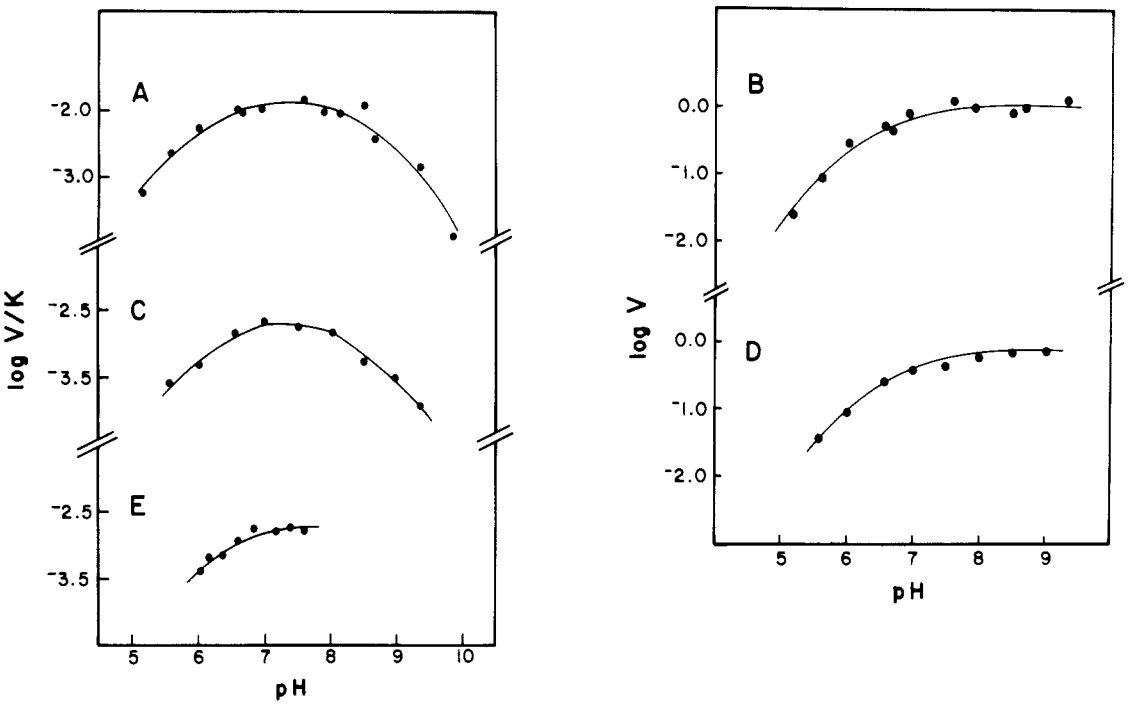


FIGURE 3: pH profiles for prephenate and deoxoprephenate as substrates for prephenate dehydrogenase. V/K profiles for prephenate (A) and deoxoprephenate (C). V profiles for prephenate (B) and deoxoprephenate (D). (E) V/K profile for prephenate in the presence of 20% dimethyl sulfoxide.

The isotope effect calculated from eq 4 was 1.0082 ± 0.0008.

Kinetic Properties of Prephenate Analogues as Substrates for Prephenate Dehydrogenase. The initial velocity pattern resulting from varying deoxoprephenate from 45 to 270 μM at four NAD levels from 79 to 790 μM was an intersecting one, and the fitted constants are in Table II. Reciprocal plots showed some curvature (concave up) at subsaturating NAD levels, and for fitting to eq 2, the lowest velocity point was discarded.

Deoxodihydroprephenate was also a good substrate. The synthesis results in a pair of enantiomers (**12a** and the compound with the double bond on the right side of the ring), which are oxidized by prephenate dehydrogenase. These enantiomers show V/K values that differ by a factor of 23, so that NADH production from the mixture is biphasic.[4] The V/K values for fast and slow isomers were obtained by fitting the reaction time course to eq 5. An apparent V value for the fast isomer was also obtained by varying the deoxodihydroprephenate concentration at saturating NAD and fitting the initial velocities to eq 1. The available kinetic parameters for both isomers are in Table II. Kinetic parameters for the saturated analogue of deoxoprephenate with no double bonds in the ring, which is a very poor substrate, are also in Table II.

pH Dependence of V and V/K for Prephenate Dehydrogenase. $V/K_{prephenate}$ decreases at both low pH and high pH, while the V profile decreases only at low pH (Figure 3). Fitting the data in Figure 3A to eq 7 gave pK values of 6.5 ± 0.1 and 8.3 ± 0.13 for the V/K profile, while the data in Figure 3B were fitted to eq 6, giving a pK of 6.7 ± 0.1 for the V profile. With deoxoprephenate, the V/K profile is similar with pK values of 6.6 ± 0.1 and 8.0 ± 0.1, and the V profile shows only the pK of 6.9 ± 0.1 (Figure 3C,D). In the presence of 20% dimethyl sulfoxide, the pK in the V/K profile for prephenate was 6.6 ± 0.1 (Figure 3E), and since this value is not significantly different from the value in water, the enzyme group responsible for this pK is probably a cationic acid (likely histidine).

To determine the enthalpy of ionization of the catalytic group with pK 6.5, the temperature dependence of this pK was determined. At temperatures of 18.1, 25.0, and 31.8 °C, pK values of 6.60 ± 0.25, 6.50 ± 0.15, and 6.40 ± 0.30 were obtained. The pK values correspond to a ΔH_{ion} value of 6 kcal/mol (data fitted to eq 8), but in view of the size of the errors, no firm value can be obtained.

Kinetic Deuterium Isotope Effects on Prephenate Dehydrogenase Reaction. When deoxoprephenate-*4-d* and deoxoprephenate were varied at fixed NAD concentrations, isotope effects were observed on both V and $V/K_{deoxoprephenate}$.

Table I: ^{13}C Isotope Effects on Decarboxylation at 25 °C[a]

substrate	catalyst	% reaction[b]	isotope ratios (×10⁵)[c] low conversion	100% conversion	$^{13}(V/K)$[d]
		$^{13}k_{obsd}$ = 1.0082 ± 0.0008			
1	H^+	10.3	1204.40	1213.05	1.0076
		15.1	1203.70	1213.41	1.0088
		$^{13}(V/K)$ = 1.0032 ± 0.0002			
1	enzyme	12.0	1212.02	1215.55	1.0031
		26.3	1212.09	1215.63	1.0034
		$^{13}(V/K)$ = 1.0033 ± 0.0008			
9	enzyme	23.1	1174.30	1177.03	1.0027
		35.0	1174.49	1178.09	1.0038
		$^{13}(V/K)$ = 1.0103 ± 0.0013			
9-*d*	enzyme	24.1	1167.53	1177.07	1.0094
		24.1	1166.20	1177.53	1.0112

[a] Enzymatic reactions at pH 7.65; acid-catalyzed decarboxylation at pH 5.15. [b] Values for the low-conversion samples determined by recovery of CO_2 from acidified reaction mixtures. [c] Ratios were adjusted for ^{17}O contribution to m/e 45 by subtracting 74 from decade settings for m/e 45/44, which were corrected to tank standard of 1260 (O'Leary, 1980). [d] Calculated from eq 4.

[4] This type of discrimination is also seen with liver alcohol dehydrogenase, which shows a biphasic time course with 2-cyclohexen-1-ol with a 5-fold difference in V/K values for fast and slow isomers. The initial velocities gave a K_m of 180 μM and a V 68% that of ethanol at saturating (0.76 mM) NAD, pH 8.0.

Table II: Kinetic Properties of Prephenate Analogues as Substrates for Prephenate Dehydrogenase at pH 8, 25 °C

substrate	K_{NAD} (μM)	$K_{i\,NAD}$ (μM)	$K_{substrate}$ (μM)	$K_{i\,substrate}$ (μM)	relative V	relative V/K
1	180 ± 40	440 ± 130	36 ± 5	90 ± 15	(100)	(100)
9	84 ± 43	320 ± 74	156 ± 33	600 ± 330	78	18
12a fast[a]	ND[b]	ND	280 ± 31	ND	5.3	0.7
12a slow	ND	ND	ND	ND	ND	0.03
17	ND	ND	1500–6000[c]	ND	0.07	10^{-5}

[a] The deoxodihydroprephenate enantiomer having the greater V/K value. [b] Not determined. [c] The concentration of the active isomer of **17** was not precisely known since it was not practical to perform enzymatic concentration calibrations with this very slow substrate.

Table III: Primary Deuterium Isotope Effects for Prephenate Dehydrogenase at 25 °C

substrate	pH	[NAD] (mM)	^{D}V	$^{D}(V/K_{substrate})$
9	8.0	2.2	1.34 ± 0.25	2.38 ± 0.27
9	8.0	0.8	1.59 ± 0.21	2.34 ± 0.12
9	5.7	0.41	1.73 ± 0.18	2.03 ± 0.16
12a fast	8.0	1.0	2.24 ± 0.26[a]	3.06 ± 0.18[a]
				3.57 ± 0.05[b]
12a slow	8.0	1.0		4.07 ± 0.12[b]

[a] Deuterium isotope effect determined by direct comparison of the initial velocities. [b] Deuterium isotope effect determined by an analysis of the biphasic time course with eq 5.

The data were fitted to eq 3, and the isotope effects are in Table III. The deuterium isotope effects for the oxidation of deoxodihydroprephenate were determined in two ways. By an analysis of the biphasic time courses with eq 5, V/K isotope effects of 3.57 ± 0.05 and 4.07 ± 0.12 were calculated for the fast and slow isomers. A direct comparison of initial velocities with deuterated and unlabeled substrate gave isotope effects of 3.06 ± 0.18 on V/K and 2.34 ± 0.26 on V for the fast isomer. These isotope effects are also summarized in Table III.

^{13}C Kinetic Isotope Effects on Prephenate Dehydrogenase Reaction. The $^{13}(V/K_{prephenate})$ and $^{13}(V/K_{deoxoprephenate\text{-}4\text{-}d})$ and $^{13}(V/K_{deoxoprephenate})$ isotope effects were determined from a comparison of the $^{13}C/^{12}C$ ratios in CO_2 from partial and complete reactions. These ratios, together with the isotope effects calculated by the use of eq 4, are in Table I.

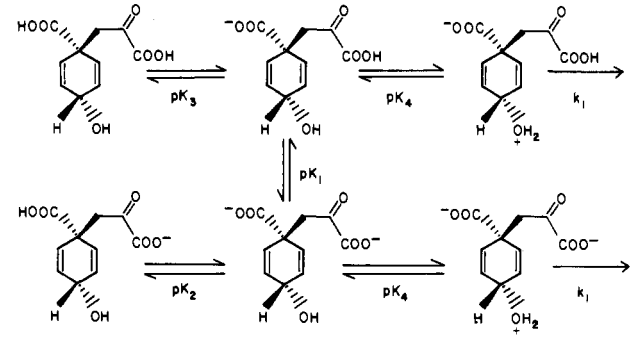
Product of Reaction with Deoxodihydroprephenate. When an experiment similar to those described for isolation of CO_2 from the prephenate dehydrogenase catalyzed oxidations of prephenate and deoxoprephenate was performed with deoxodihydroprephenate, no CO_2 could be isolated. Amounts of 50 mg (50 μmol of the fast isomer) of deoxodihydroprephenate, 10 mg (90 μmol) of sodium pyruvate, and 8 mg (11 μmol) of NAD were dissolved in 3.0 mL of 100 mM Hepes, pH 7.8, containing 1 mM each of dithiothreitol and EDTA. Prephenate dehydrogenase (9.4 units) and 1000 units of lyophilized lactate dehydrogenase were added, and after 3 h, 0.6 mL of 1 N HCl was added (pH was 2), and the aqueous solution was extracted with 3 mL of diethyl ether. A thin-layer chromatogram of this solution on silica gel 60 (F-254) revealed a fluorescent quenching spot with an R_f identical with that of authentic 1-carboxy-4-oxo-2-cyclohexene-1-propanoic acid (**13**) [R_f (toluene/acetic acid, 12:7) 0.33]. The ether solution was then extracted into 0.01 N NaOD/D_2O and lyophilized (pD 8.5). The residue was dissolved in 99.8% D_2O, lyophilized, and taken up in 0.5 mL of 100.00% D_2O. The proton NMR spectrum contained peaks identical with those of **13**, plus the resonances corresponding to the enzymatically inactive isomers of deoxodihydroprephenate.

Alternate Nucleotides as Substrates. 3-Acetylpyridine–NAD and 3-pyridinecarboxaldehyde–NAD were both good substrates for prephenate dehydrogenase with V values 107 and 13% that of NAD. The other kinetic parameters determined from initial velocity patterns are shown in Table IV.

Table IV: Kinetic Properties of Nucleotide Substrates for Prephenate Dehydrogenase at pH 8, 25 °C

nucleotide	$K_{nucleotide}$ (μM)	$K_{prephenate}$ (μM)	relative V	relative $V/K_{prephenate}$
NAD	180 ± 40	36 ± 5	(100)	(100)
acetylpyridine–NAD	210 ± 98	132 ± 34	107	40
pyridinecarboxaldehyde–NAD	200 ± 70	75 ± 31	13	9

Scheme IV



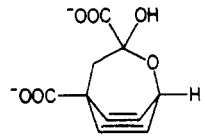
Nucleotide Side Specificity. When NAD-*4-d* was reduced with prephenate by using prephenate dehydrogenase and reoxidized with either glutamate dehydrogenase (B face) or lactate dehydrogenase (A face), proton NMR spectra showed that in the former case deuterium was still present at C-4 but that with lactate dehydrogenase a proton was now present at C-4. Prephenate dehydrogenase must therefore catalyze the transfer of hydride to the B or *si* face of NAD.

Discussion

Acid-Catalyzed Decarboxylation. The pH dependences of the rates of prephenate and deoxoprephenate decomposition in acid (Figure 1) can be interpreted in terms of Scheme IV. The net rate constant for decarboxylation of the reverse protonation species, k_1, corresponds to more than a single step, as we will discuss later in more detail. For both prephenate and deoxoprephenate, pK_4 is assumed to be very low and independent of the protonation state of the remote carboxyl group.[5] The profile for prephenate (Figure 1A) shows pK values of 1.4, 4.2, and 3.7 for pK_1, pK_2, and pK_3, respectively. The profile for deoxoprephenate (Figure 1B) shows only a single pK of 4.3, since pK_1 and pK_2 are nearly identical for this analogue (the keto group lowers pK_1 considerably in prephenate).

[5] This may not be a valid assumption, but the change in pK_2 upon protonation of the remote carboxyl is clearly larger than that in pK_4, and $pK_2 - pK_3$ really represents the degree by which pK_2 is more affected than pK_4.

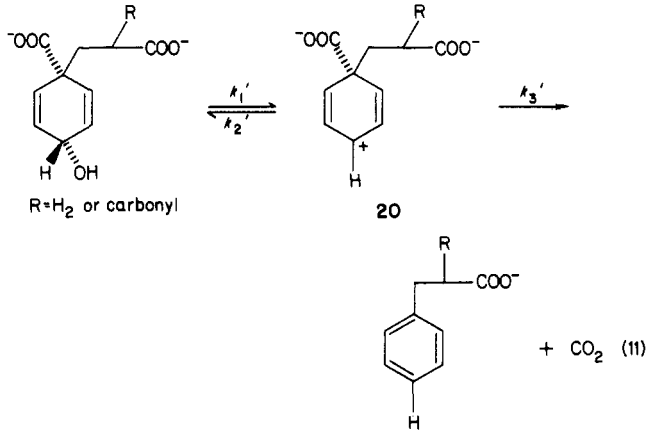
The deoxoprephenate used in these experiments was a mixture of isomers **9** and **10**. ^{13}C NMR spectra of the mixture and of a sample that had been incubated with prephenate dehydrogenase allowed assignment of the individual resonances to either **9** or **10**. ^{13}C NMR analysis showed that **9** and **10** decomposed in acid at identical rates, in contrast with prephenate, where the epi isomer decarboxylates in acid at less than 10% the rate of the natural isomer. We believe that the acid stability of *epi*-prephenate is due to the formation of an internal hemiketal (**19**).[6] This structure explains why the ^{13}C



19

chemical shift of C-4 in *epi*-prephenate (64.6 ppm) is displaced from that of prephenate (62.4 ppm) or from those of deoxoprephenate (62.8 ppm) and its epi isomer (62.7 ppm). Prephenate contains a large variety of organic functionality within a small carbon skeleton. Nature's use of the trans relationship between the pyruvyl side chain and the hydroxyl group in prephenate avoids intramolecular reaction of these functionalities.

The steps grouped together as k_1 in Scheme IV were characterized by ^{13}C NMR analysis of partial reactions in $H_2{}^{18}O$. Since ^{18}O could be detected in residual prephenate and deoxoprephenate after 67–85% reaction (Figure 2), the acid-catalyzed decarboxylation must proceed through a resonance-stabilized carbonium ion (**20**) whose lifetime is long enough to allow the unlabeled H_2O produced to exchange with the $H_2{}^{18}O$ solvent:



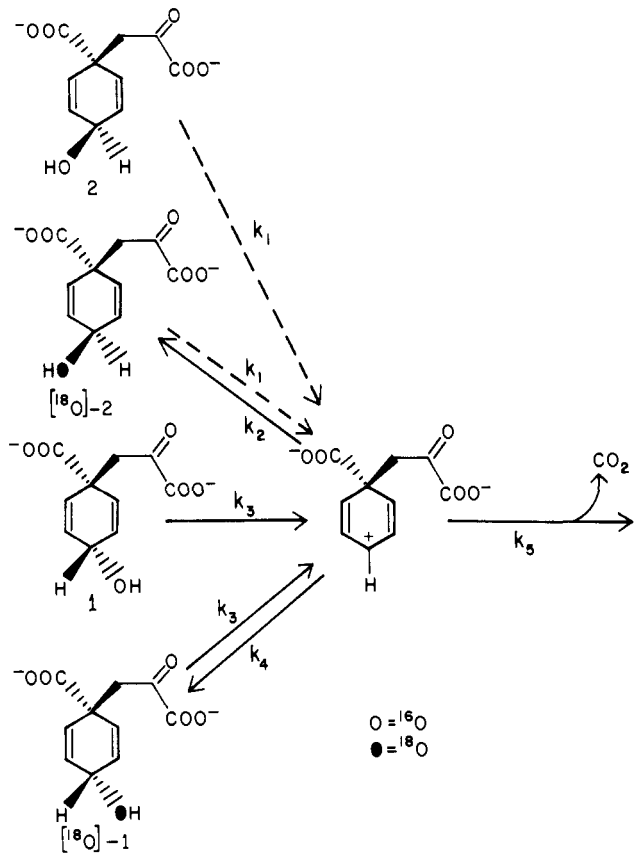
R=H_2 or carbonyl

20

+ CO_2 (11)

Once the planar carbonium ion is formed, the addition of water can occur at either face. A more complete kinetic scheme for the acid-catalyzed decarboxylation of prephenate is shown in Scheme V, where the reaction was begun with 1 and a small contaminant of **2** (Figure 2). Solution of the differential equations that describe the reactions in Scheme V is made much simpler by assuming that k_1 of Scheme V is equal to zero (that is, that *epi*-prephenate is not acid labile during the experiment). Since the ^{13}C NMR peak corresponding to **2** did not change in intensity by more than 10% over the course

Scheme V



of the reaction, this assumption is justified. The following equations then describe the reactant concentrations at fraction f of disappearance of **1**, where A, B, C, and D represent the concentrations of **2**, **2**-^{18}O, **1**, and **1**-^{18}O, respectively:

$$B/C_0 = (1 - e^{xy})/(1 + k_5/k_2) \quad (12)$$

$$D/C_0 = e^{xy} - e^{y} \quad (13)$$

$$C/C_0 = e^{y} \quad (14)$$

where

$$x = (1 + k_5/k_2)/(1 + k_4/k_2 + k_5/k_2) \quad (15)$$

$$y = -\ln (1 - f) \quad (16)$$

Thus

$$k_5/k_2 = (1 - D/C_0 - C/C_0)/(B/C_0) - 1 \quad (17)$$

$$k_4/k_2 = (1 + k_5/k_2)(y/[\ln (D/C_0 + C/C_0)] - 1) \quad (18)$$

From the integrations of the peaks in Figure 2B, k_5/k_2 and k_4/k_2 were 4.9 and 1.8, respectively. Thus, rehydration of **20** is ~2-fold faster on the face which yields the natural isomer than on that which yields *epi*-prephenate.

In contrast to prephenate, both isomers of deoxoprephenate decarboxylate at identical rates, and ^{18}O is incorporated into each isomer to the same extent. The simple scheme of eq 11 can thus be used for determining the partitioning of the carbonium ion intermediate. The quantitative relationship between deoxoprephenate (A), the ^{18}O-labeled species (B), the fraction f of disappearance of A, and the ratio k_3'/k_2' is

$$B/A = e^{y/(1+k_3'/k_2')} - 1 \quad (19)$$

where y is given by eq 16. Thus

$$k_3'/k_2' = y \ln (B/A + 1) - 1 \quad (20)$$

[6] Pleininger (1962) noted that the cis and trans isomers of 1-carboxy-4-hydroxy-2,5-cyclohexadiene-1-acetic acid decomposed at pH 2.8 with half-lives of 38.4 and 1.7 min, respectively. Presumably, the cis isomer (hydroxyl and acetic acid functionality on the same side of the ring) exists largely as a lactone at this pH, thus slowing down decarboxylation.

From the integrations of the peaks in Figure 2D, k_3'/k_2' equals 2.6 for deoxoprephenate.

The ^{13}C isotope effect ($^{13}k_{obsd}$) determined for the acid-catalyzed decarboxylation of prephenate was 1.0082 (Table I), and is given by

$$^{13}k_{obsd} = (^{13}k_5 + c_f)/(1 + c_f) \quad (21)$$

where

$$c_f = k_5/(k_2 + k_4) = (k_5/k_2)(1 + k_4/k_2) \quad (22)$$

The estimates for k_5/k_2 and k_4/k_2 of 4.9 and 1.8 obtained from the ^{18}O incorporation experiment allow calculation of the forward commitment, c_f, as 1.75 and the intrinsic ^{13}C isotope effect, $^{13}k_5$, on the decarboxylation step as 1.023. This value is quite small and suggests an early transition state for C–C bond cleavage.

The prephenate analogue that is partially saturated, deoxodihydroprephenate (**12**), is acid stable. The resonance stabilization of the carbonium ion that occurs with prephenate and deoxoprephenate does not occur with this analogue, and a concerted pathway for decarboxylation is apparently of much higher energy.

Enzymatic Oxidative Decarboxylation. The stepwise (Scheme Ia) and concerted (Scheme Ib) mechanisms for the oxidative decarboxylation of prephenate catalyzed by prephenate dehydrogenase are easily distinguished by the multiple isotope effect methodology developed in this laboratory. By determining the ^{13}C isotope effect on V/K with both a deuterated and an unlabeled substrate and the deuterium isotope effect on V/K, it is possible to tell whether the ^{13}C-sensitive and deuterium-sensitive steps are the same or not and, if they are different, to determine which comes first in the mechanism (Hermes et al., 1982). The ^{13}C isotope effect of interest is that originating from the C–C cleavage resulting in decarboxylation. The natural abundance of ^{13}C is conveniently used as a trace label since the product CO_2 is readily analyzed by isotope ratio mass spectrometry. Measurement of the deuterium isotope effect resulting from C–H bond cleavage at C-4 of the prephenate, on the other hand, requires specifically deuterated substrate. For synthetic reasons, we chose to evaluate the isotope effects for the more readily deuterated substrate, deoxoprephenate (**9**). This analogue is missing the keto group of the pyruvyl side chain but otherwise is identical with prephenate and has comparable kinetic parameters (Table II); the increase in K_m is presumably caused by lack of specific binding to the keto group.

Since the ^{13}C isotope effect was less with unlabeled than with deuterated deoxoprephenate, both the ^{13}C and the deuterium isotope effects are on the same step (that is, deuteration has made the ^{13}C-sensitive step more rate limiting by slowing it down). If the mechanism had been stepwise, the ^{13}C isotope effect would have been less with the deuterated substrate, since deuteration would slow down a step other than the ^{13}C-sensitive one.[7]

[7] It could be argued that in the stepwise mechanism of Scheme Ia there would be an equilibrium secondary ^{13}C isotope effect on the first step as the result of loosening of the bond from the carboxyl group to the ring, in addition to the primary isotope effect on the decarboxylation step. From calculations we have discovered that, for the observed data to be consistent with this model, the secondary ^{13}C kinetic isotope effect on the first step would have to be ~1.0155 and the partition ratio of the ketone intermediate between decarboxylation and reverse hydride transfer would have to be very large. A secondary ^{13}C isotope effect this large corresponds, however, to a partly broken C–C bond, and thus, we feel that such a "stepwise" model is not semantically different from a concerted one in which C–C bond breaking is less advanced than C–H cleavage in the transition state.

Table V: Intrinsic Isotope Effects and Commitments for Reaction of Deoxoprephenate with Prephenate Dehydrogenase[a]

assumed c_r/c_f	c_f	c_r	^Dk	^{13}k
0	3.70	0	7.3	1.0155
0.3	5.47	1.64	11.6	1.0158
1.0	−129	−129	−319	1.0162
3.0	−1.9	−5.8	−7.0	1.0167
∞	0	−4.1	−2.5	1.0174

[a] Experimental parameters were as follows: $^{13}(V/K)_H = 1.0033 \pm 0.0008$; $^{13}(V/K)_D = 1.0103 \pm 0.0013$; $^{13}K_{eq} = 1.0067$ [estimated from Cleland (1980)]; $^D(V/K) = 2.34 \pm 0.12$; $^DK_{eq} = 1.18 \pm 0.02$ (Cook et al., 1980). The values tabulated were calculated with eq 23–25 by the method of Hermes et al. (1982).

In addition to establishing the concerted nature of the prephenate dehydrogenase reaction, the determination of $^{13}(V/K)_H$, $^{13}(V/K)_D$, and $^D(V/K)$ allows calculation of limits on the intrinsic ^{13}C and deuterium isotope effects and the commitments in the reaction (Hermes et al., 1982). Equations 23–25 describe these observed isotope effects, where ^{13}k and

$$^{13}(V/K)_H = \frac{^{13}k + c_f + c_r{}^{13}K_{eq}}{1 + c_f + c_r} \quad (23)$$

$$^{13}(V/K)_D = \frac{^{13}k + c_f/{}^Dk + c_r{}^DK_{eq}{}^{13}K_{eq}/{}^Dk}{1 + c_f/{}^Dk + c_r{}^DK_{eq}/{}^Dk} \quad (24)$$

$$^D(V/K) = \frac{^Dk + c_f + c_r{}^DK_{eq}}{1 + c_f + c_r} \quad (25)$$

^Dk are the intrinsic isotope effects on the bond-breaking step, c_f and c_r are forward and reverse commitments, and $^{13}K_{eq}$ and $^DK_{eq}$ are equilibrium isotope effects. Note that in eq 24 that deuteration of the substrate has decreased the commitments by slowing down the bond-breaking step by ^Dk in the forward direction and $^Dk/^DK_{eq}$ (the reverse intrinsic deuterium isotope effect) in the reverse direction. These equations can be solved simultaneously for ^Dk and ^{13}k for a range of assumed c_r/c_f ratios from zero to infinity, and these solutions are in Table V. With no further assumptions, a very narrow range of possible ^{13}k values is obtained, since negative values of c_f, c_r, or ^Dk are impossible. The reverse commitment for this reaction (the ratio of the rate of reverse hydride transfer from NADH to aromatic product and the rate of CO_2 release from the enzyme) is certainly very small. Assuming that c_r equals zero, the following values can be calculated, with errors determined by the procedure of Hermes et al. (1982): $^Dk = 7.3 \pm 2.0$; $^{13}k = 1.0155 \pm 0.0024$; $c_f = 3.7 \pm 1.4$. These values suggest that in the transition state the hydride is roughly symmetrically placed between C-4 of deoxoprephenate and C-4 of NAD but the bond between C-1 and the carboxyl group is only slightly stretched, so that for C–C bond cleavage the transition state is very early, as would be expected for an exothermic decarboxylation in a hydrophobic environment.

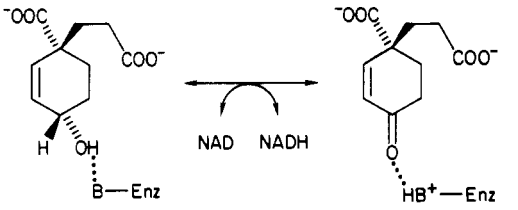
Since the experimental evidence suggests carbonium ion **20** as an intermediate in the acid-catalyzed reaction, what is the reason for the concerted nature of the prephenate dehydrogenase catalyzed decarboxylation? In aqueous solution strong hydrogen bonds form between the carboxyl group of **20** and water, and in a concerted mechanism these hydrogen bonds would have to be broken simultaneously with C–C and C–O bond cleavages. On the enzyme, however, the carboxyl group is presumably in a hydrophobic pocket to promote decarboxylation. Under these conditions, the lifetime of the dienone becomes so short that C–C cleavage occurs in the same transition state as, though not synchronous with, the hydride transfer.

The pH variation of V and V/K (Figure 3) for deoxoprephenate is very interesting in light of this concerted behavior. The decrease in both V and V/K below pH 6.7 is the signature of a catalytic group (Cleland, 1977). Apparently, even though the proton is never fully transferred to this base from the hydroxyl group of prephenate, the polarization of the O–H bond is necessary for hydride abstraction by NAD to begin (the transfer is complete in the oxidation of **12** or **17**; see below). This is evident in both the pH profiles and the observation that the prephenate analogue missing this hydroxyl is not a substrate (J. F. Morrison, unpublished experiments). The absence of a solvent perturbation on this pK by 20% dimethyl sulfoxide and the low enthalpy of ionization (~6 kcal/mol) imply that the catalytic group is the imidazole of a histidine residue. At high pH a decrease is seen in the V/K but not in the V profile. This behavior is characteristic of an amino acid involved in substrate binding; possibly this pK is that of a histidine or lysine responsible for binding of the remote carboxyl group of the substrate.

Substrates in enzymatic reactions are "sticky" if they react to give products as fast or faster than they dissociate from the enzyme. If a substrate is sticky and it is possible for the catalytic group to be incorrectly protonated, the pK seen in the V/K profile will be displaced toward lower pH when the group has to be ionized for activity. Prephenate and deoxoprephenate have greater than a 5-fold difference in $V/(KE_t)$ values but nearly identical maximum velocities. Since the pK values in the V/K profiles for these two substrates (Figure 3) are not significantly different, it appears that neither is sticky. If deoxoprephenate were sticky, the deuterium isotope effect on V/K would increase at low pH, but it actually decreased slightly, showing that the forward commitment of 3.7 is all internal and deoxoprephenate is not sticky. This commitment may represent the conformation change in the enzyme after substrate is bound that deforms the pyridine ring of NAD to develop carbonium ion character at C-4 and induce hydride transfer (Cook et al., 1981).

The deuterium isotope effect with deoxoprephenate was also determined at two NAD concentrations. Since it did not decrease when the NAD concentration was raised, a steady-state ordered kinetic mechanism with prephenate adding first is ruled out (Cook & Cleland, 1981). Previous studies by Sampathkumar & Morrison (1982) with NAD-^{14}C have shown that NAD cannot be trapped in an isotope partition experiment, thus eliminating a steady-state ordered mechanism with NAD adding prior to prephenate. It appears that the kinetic mechanism is random.

Four other enzymes that catalyze NAD(P)-dependent oxidative decarboxylations (NADP- and NAD-dependent malic enzymes, isocitrate dehydrogenase, and 6-phosphogluconate dehydrogenase) all catalyze stepwise reactions in which a β-keto acid intermediate is formed prior to decarboxylation, although these reactions are all freely reversible (Hermes et al., 1982; P. F. Cook, D. Kiick, B. Harris, and J. D. Hermes, unpublished results; Grissom & Cleland, 1983; Rendina et al., 1984). The reasons for the concerted nature of the prephenate dehydrogenase reaction were thus of interest. To determine whether this unprecedented mechanism was due simply to the unique nature of the substrate and its ensuing aromatic product (that is, to the irreversibility of the reaction), the mechanism with deoxodihydroprephenate (**12a**) was investgated. The primary deuterium isotope effects for both active isomers of deoxodihydroprephenate (**12a** and its enantiomer) are larger than those observed with deoxoprephenate, as is often seen with a slow substrate when the chemical transformation responsible for the isotope effect is more rate limiting. This substrate is partially saturated, and thus, the reaction course catalyzed by prephenate dehydrogenase is not influenced by any stabilization provided by aromatization. Although it is a good substrate for oxidation, deoxodihydroprephenate is *not* decarboxylated but simply oxidized in a reversible reaction to 1-carboxy-4-oxo-2-cyclohexene-1-propanoate (**13**):



13

This mechanism has changed from concerted with deoxoprephenate to stewise with deoxodihydroprephenate in the extreme sense that the second step never occurs and **13** is released into solution. Prephenate dehydrogenase is clearly not designed to decarboxylate **13**, since during reaction with the natural substrate no such intermediate exists. With prephenate (or deoxoprephenate), prephenate dehydrogeanse must thus provide only the catalytic properties associated with a simple alcohol dehydrogenase, and the nature of the substrate dictates the decarboxylation. This study illustrates the manner in which substrate chemistry can control the reaction mechanism in enzymatic reactions.

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Carbon Isotope Effects on the Enzyme-Catalyzed Carboxylation of Ribulose Bisphosphate†

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ABSTRACT: ^{13}C isotope effects associated with the carboxylation of ribulose bisphosphate by ribulosebisphosphate carboxylase from spinach have been measured by comparison of the isotopic composition of carbon 1 of the 3-phosphoglyceric acid formed with that of the CO_2 substrate. Correction was made for the 3-phosphoglyceric acid that is formed from carbons 3–5 of ribulose bisphosphate. The carbon isotope effect is $k^{12}/k^{13} = 1.029 \pm 0.001$ at pH 8.0, 25 °C. The same isotope effect is observed in the presence of either 1 or 0.2 mM ribulose bisphosphate. Deuteration of carbon 3 of the substrate decreases the carbon isotope effect to 1.021. At pH 9.0, the isotope effect is 1.026, whereas at pH 7.0 it is 1.030. These results are consistent with the generally accepted mechanism for the carboxylation involving enolization of ribulose bisphosphate followed by carboxylation, provided that CO_2 binds to the enzyme prior to the enolization. Carboxylation is principally, though not entirely, rate limiting. The enzyme probably catalyzes hydrogen exchange between solvent and substrate. The isotope effects do not provide an unambiguous choice between random and ordered mechanisms of substrate addition.

Ribulosebisphosphate carboxylase-oxygenase (EC 4.1.1.39), the major enzyme responsible for CO_2 fixation by plants (Miziorko & Lorimer, 1983; Lorimer, 1981a; Akazawa, 1979), catalyzes the carboxylation and oxygenation of RuBP (Figure 1). The accepted mechanism of carboxylation of RuBP,[1] first suggested by Calvin (1956), is shown in Figure 2. Carbon dioxide reacts with an enol or enolate derived from RuBP to form an enzyme-bound, six-carbon intermediate, which is cleaved by reaction with water to form two molecules of 3-PGA. When the carboxylation is conducted in labeled water, label is introduced at C-2 of one of the two 3-PGA molecules (Mullhofer & Rose, 1965; Hurwitz et al., 1956) and at C-3 of recovered RuBP (Saver & Knowles, 1982). 4-Carboxyarabinitol bisphosphate and 2-carboxyarabinitol bisphosphate, analogues of the putative six-carbon intermediate, are tight-binding inhibitors of the enzyme (Schloss & Lorimer, 1982; Pierce et al., 1980; Siegel & Lane, 1972). Recent studies of the six-carbon intermediate indicate that the enzyme-catalyzed cleavage of this intermediate to enzyme-bound 3-PGA is favored over cleavage to enolate and CO_2 by at least a factor of 10 (G. Lorimer and J. V. Schloss, personal communication). Isotope-labeling studies mitigate against formation of a covalent enzyme–RuBP complex (Lorimer, 1978; Sue & Knowles, 1978).

CO_2, rather than HCO_3^-, is the substrate (Cooper et al., 1969). Although the reaction shows saturation kinetics with respect to CO_2 (Badger & Andrews, 1974), this does not necessarily indicate that CO_2 binds to the enzyme prior to reaction with the enolate. Oxygenation of RuBP appears to occur by an analogous mechanism in which the enol or enolate reacts with O_2, rather than CO_2. Kinetically, the reaction also shows saturation kinetics with respect to O_2.

Both carboxylase (Lorimer et al., 1976) and oxygenase (Badger & Lorimer, 1976) require activation by CO_2 and a divalent metal. Kinetic studies of the activation have led to the two-step mechanism

$$E \xrightleftharpoons[\text{slow}]{CO_2} E\text{—}\underset{H}{N}\text{—}CO_2^- \xrightleftharpoons[\text{fast}]{M^{2+}} E\text{—}\underset{H}{N}\text{—}CO_2^-M^{2+} \quad (1)$$

This "activating" CO_2 is distinct from "substrate" CO_2 (Miziorko, 1979; Lorimer, 1979). Formation of a carbamate during activation has been demonstrated by ^{13}C NMR (O'Leary et al., 1979) and by isolation of a methyl ester derivative of the carbamate following treatment of the stable $^{14}CO_2$–Mg^{2+}–2-carboxyarabinitol bisphosphate complex with diazomethane (Lorimer & Miziorko, 1980). Peptides containing N^{ϵ}-(methoxycarbonyl)lysine have been sequenced (Lorimer, 1981b; Donnelly et al., 1983).

The enzyme can be assayed by a radiochemical procedure or by a complex coupled assay (Racker, 1974). Measured activities are often time dependent. The complexity of the assays and the activation requirement of the enzyme seem to have had a dampening effect on kinetic studies. One of the most serious difficulties encountered is the presence of inhibitors in the RuBP used. The best characterized of these is xylulose 1,5-bisphosphate, the C-3 epimer of RuBP (McCurry & Tolbert, 1977; Paech et al., 1978), which is a good inhibitor ($K_i \approx 3$ μM) and is generated in situ from RuBP. This inhibitor can be removed (with difficulty) by

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[1] Abbreviations: CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; di-PGA, 2,3-diphosphoglyceric acid; HEPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; 3-PGA, 3-phosphoglyceric acid; RuBP, ribulose 1,5-bisphosphate; XuBP, xylulose 1,5-bisphosphate; EDTA, ethylenediaminetetraacetic acid; Bicine, *N,N*-bis(2-hydroxyethyl)glycine.

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