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Enzymatic and Nonenzymatic Dehydration Reactions of L-Arogenate†

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ABSTRACT: L-Arogenate, an immediate precursor of either L-tyrosine, L-phenylalanine, or both in many microorganisms and plants, may undergo two types of dehydration reactions that yield products of increased stability. Under acidic conditions, a facile aromatization attended by loss of the C-4 hydroxyl and the C-1 carboxyl moieties results in quantitative conversion to L-phenylalanine. When aromatization was largely prevented by maintaining pH in the range of 7.5–12, a second dehydration reaction occurred in which the alanyl side chain and the carboxyl group at C-1 formed a lactam ring to yield spiro-arogenate. The latter reaction occurs at 100 °C, roughly 50% conversion being obtained in 2 h. The product formed from L-arogenate was authentic spiro-arogenate, as demonstrated by high-performance liquid chromatography and thin-layer chromatography identification procedures. Further confirmation was obtained by ^1H nuclear magnetic resonance, ultraviolet spectroscopy, and mass spectrometry. Thus far, the conversion of L-arogenate to spiro-arogenate is not known to be enzyme catalyzed. The other dehydratase reaction, however, is catalyzed in nature by an enzyme denoted arogenate dehydratase. An improved assay is described for this in which [^3H]dansyl derivatives of L-arogenate (substrate) and L-phenylalanine (product) are separated by using bidimensional thin-layer chromatography. The radioactive reaction product is then quantitated. This assay was used to study partially purified arogenate dehydratase from *Pseudomonas diminuta*, an organism that depends upon the arogenate pathway for L-phenylalanine biosynthesis. This enzyme possessed a $K_{m,app}$ of 0.63 mM for L-arogenate and was sensitive to inhibition by L-phenylalanine, 50% inhibition being obtained at 70 μM L-phenylalanine. In contrast, arogenate dehydratase isolated from *Pseudomonas aeruginosa*, an organism that possesses both the phenylpyruvate and arogenate routes to L-phenylalanine, was insensitive to inhibition by L-phenylalanine.

L-(8*S*)-Arogenate has taken on steadily increasing significance as an amino acid precursor of L-tyrosine, L-phenylalanine, or both in prokaryotic and eukaryotic microorganisms, as well as in higher plants (Byng et al., 1982). Zamir et al. (1980) showed the structure of L-arogenate to be β-(1-carboxy-4-hydroxy-2,5-cyclohexadien-1-yl)alanine, the expected transamination product of prephenate. A second cyclohexadienyl structure, isolated by Zamir et al. (1983c) from culture supernatants of *Neurospora crassa*, is the lactam derivative of L-arogenate and was assigned the trivial name spiro-arogenate (spiro[4-hydroxy-2,5-cyclohexadiene-1,γ-L-pyroglutamic acid]).

Under acidic conditions both compounds are aromatized following dehydration to yield L-phenylalanine. At a given pH, the rate of aromatization increases in proportion to elevation of reaction temperature. Acid-catalyzed conversion of spiro-arogenate to L-phenylalanine undoubtedly progresses through a step in which L-arogenate is formed as an intermediate. Figure 1 illustrates a feasible mechanism for acid-catalyzed aromatization of arogenate, a sequence in which the C-4 hydroxyl group is protonated, leading to extrusion of the carboxyl moiety and elimination of water to yield a stable aromatic ring.

We have recently found that at mildly alkaline pH where decarboxylation of L-arogenate is largely prevented, a second type of dehydration reaction, yielding spiro-arogenate, will occur at elevated temperature. The dehydratase reaction that aromatizes L-arogenate is already established (Byng et al.,

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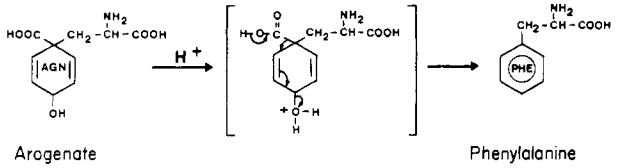

FIGURE 1: Probable mechanism for the acid-catalyzed aromatization of L-arogenate to form L-phenylalanine.

1982) as a major enzyme activity (arogenate dehydratase), while the second (lactam-yielding) dehydratase reaction has only been speculated (Zamir et al., 1983c) to be enzyme catalyzed (spirase). The enzymatic and nonenzymatic dehydratase reactions which may occur with L-arogenate are described in this paper.

Materials and Methods

Materials. Amino acids, *p*-fluoro-DL-phenylalanine, β-2-thienyl-DL-alanine, Sephadex G-25, and bovine serum albumin were from Sigma Chemical Co. DEAE-cellulose (DE-52) was from Whatman. Aquasol, scintillation vials, and [^{3}H]dansyl chloride in acetone were obtained from New England Nuclear Co. L-Arogenate was purified as described by Zamir et al. (1980), as modified by Zamir et al. (1983a). Spiro-arogenate was prepared as described by Zamir et al. (1983c). Polyamide plates for thin-layer chromatography were purchased from Pierce.

Microbiological Aspects. Pseudomonas diminuta strains ATCC 13184 and ATCC 11568 were obtained from the American Type Culture Collection. *Pseudomonas aeruginosa* ATCC 15692, maintained in our laboratories for over 15 years, was originally obtained from B. Holloway as strain PAO 1. *P. diminuta* was grown at 26 °C in Difco nutrient broth medium. *P. aeruginosa* was grown at 37 °C in a minimal salts medium (Calhoun & Jensen, 1972) containing 0.5% (w/v) glucose but lacking sodium citrate. Cell pellets used for preparation of enzyme extracts were obtained by centrifugation of cultures in late-exponential growth.

Preparation of Crude Extracts. Whole-cell pellets were resuspended in 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol, and several bursts of sonic energy were applied at 4 °C by using a Lab-line ultratip sonicator. Cellular debris was removed following centrifugation at 150000*g* for 1 h. The resulting extract was desalted by passage through a Sephadex G-25 column equilibrated in 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol, and this preparation was denoted as crude extract.

Column Chromatography. Partial purification of arogenate dehydratase from *P. diminuta* ATCC 13184 was carried out at 4 °C by loading 600 mg of crude extract protein onto a DEAE-cellulose column (2.5 × 40 cm) equilibrated with 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol. The column was washed with 400 mL of equilibration buffer prior to application of a 1600-mL linear salt gradient (0–0.5 M KCl). Each reservoir contained the equilibration buffer. Fractions of 8.2 mL volume were collected. The three subforms of arogenate dehydratase from *P. aeruginosa* were separated as described by Patel et al. (1977).

Assay for Arogenate Dehydratase. Two assays were used to estimate arogenate dehydratase activity. A method in which unutilized L-arogenate (substrate) is selectively oxidized by potassium permanganate under conditions in which L-phenylalanine (product) remains intact was described by Shapiro et al. (1980). With this technique, the fluorescence of a phenylalanine–dipeptide conjugate is measured.

A second assay technique has not previously been published. The substrate and product of the arogenate dehydratase reaction can be converted to their dansyl derivatives following treatment with dansyl chloride. The migration positions of the resulting dansylarogenate and dansylphenylalanine on thin-layer chromatograms (TLC) are readily visualized as separate fluorescent spots. If radioactive dansyl chloride is used, then the formation of labeled product can be measured with excellent accuracy and sensitivity. The following general procedure is used. A 200-μL reaction mixture containing about 1.5 mM L-arogenate and enzyme in 50 mM potassium phosphate buffer (pH 7.0) is incubated at 37 °C for 45 min. The reaction is terminated by addition of 20 μL of 2 N NaOH followed by vortexing of the mixture. A 25-μL portion is transferred to a 6 × 50 mm culture tube, and 10 μL of a 9:1 solution of [^{3}H]dansyl chloride (1 mCi/mL; 24.9 Ci/mmol)–unlabeled dansyl chloride (5% w/w in acetone) is added. Each tube is mixed, sealed with parafilm, and incubated at 37 °C for 30 min. After the samples are cooled to ice temperature, 5 μL of dansylphenylalanine is added to each sample as a carrier. Each sample is then freeze-dried, and the dansyl derivatives are dissolved from the residue by addition of 50 μL of cold methanol. Dansylarogenate and dansylphenylalanine are separated on thin-layer chromatograms by spotting 6 μL of the methanol sample at the origin position of a 5 × 5 cm polyamide plate, which is then developed bidimensionally. The solvent for the first development is NH_4OH–H_2O (1:4 v/v). Plates are air dried and developed in the second dimension with a solvent of benzene–acetic acid–pyridine (50:5:1 v/v/v). The dansyl derivatives are readily visualized under shortwave UV light, and the dansylphenylalanine spot is excised from the TLC plate and placed into a scintillation vial for tritium counting. A 100-μL amount of 88% formic acid followed by 500 μL of water is added in order to dissolve the polyamide. After addition of 10 mL of Aquasol, the vials are sealed, vortexed, and counted. Quantitative estimates of phenylalanine formed by arogenate dehydratase are read from a standard curve constructed with authentic [^{3}H]dansylphenylalanine.

HPLC Analysis of Inhibition of Arogenate Dehydratase by L-Phenylalanine. Reaction mixtures contained L-arogenate (ranging between 0.068 and 0.68 mM in different experiments), enzyme, and the indicated concentration of L-phenylalanine, in 50 mM potassium phosphate buffer at pH 7.5. The reaction was allowed to proceed at 37 °C for 60 min and then terminated by immersion in an ice bath. A 40-μL volume of reaction mixture was combined with 10 μL of 0.833 mM L-leucine (internal standard) followed by addition of 200 μL of OPA reagent (54 mg of *o*-phthalaldehyde in 1.0 mL of methanol plus 9 mL of 0.4 M sodium borate at pH 9.2). Derivatization was allowed to proceed for 1.5 min at 25 °C. The mixture was then injected into a 20-μL sample loop and swept through an Alltech Applied Science Lichrosorb RP-18 column (4.6 × 250 mm) by a 2:3 mixture of 20 mM sodium phosphate at pH 7.0 and methanol by using a flow rate of 0.9 mL/min. OPA derivatives of L-leucine, L-arogenate, and L-phenylalanine were detected fluorometrically as described below.

Protein Determinations. The concentration of protein in extracts was measured by the Bradford assay (1976) with bovine serum albumin for construction of standard curves.

Chemical Conversion of L-Arogenate to Spiro-arogenate. Solutions of L-arogenate were prepared at about 1 mM concentration in 10 mM sodium phosphate buffer (pH 7.5).

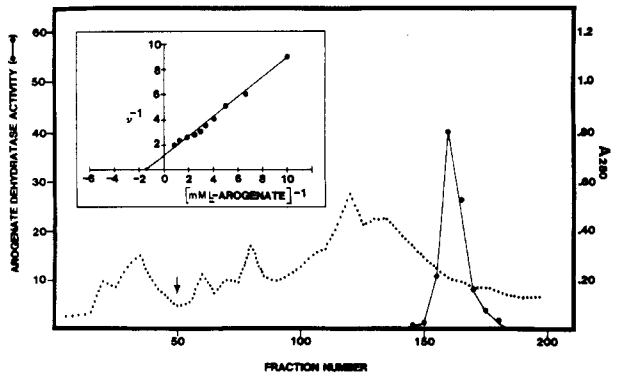

FIGURE 2: Chromatographic fractionation of arogenate dehydratase from *P. diminuta*. Crude extract was applied to DEAE-cellulose as specified under Materials and Methods. The salt gradient was initiated at fraction 50. The peak of arogenate dehydratase was located by using the oxidation–fluorescent assay; the ordinate values are nanomoles of arogenate formed per 50 μL of eluate fraction per 45 min at 37 °C. (Inset) Substrate saturation data plotted on double-reciprocal coordinates. Velocity on the ordinate scale is expressed as nanomoles per minute. Enzyme activity was measured by using the radioactive assay.

Appropriate additions of 1 N NaOH resulted in mixtures having the pH specified in the text. Such mixtures at various pH values were heated at 100 °C for up to 3 h. Other mixtures were also heated at 37 and 50 °C. Initial volumes were restored after heating by addition of distilled water. When necessary, neutralization was carried out prior to high-performance liquid chromatography (HPLC).

Aliquots of reaction mixtures were injected into a 20-μL sample loop (or 1.0-mL loop for preparative columns) and swept through one of two Altex Ultrasphere ODS (4.6 × 150 mm) analytical HPLC columns by 20 mM sodium phosphate (pH 7.2) at a flow rate of 1.0 mL/min. Solutes were detected by monitoring of ultraviolet absorbance at 215 nm on a Gilson HM UV–visible spectrophotometer. *o*-Phthalaldehyde derivatives of primary amine products were swept through the same column by a 1:1 mixture of 20 mM sodium phosphate (pH 7.0) and methanol and detected by measurement of fluorescence on a Gilson Model FL-1B fluorometer (excitation filter 360 nm; emission filter 455 nm).

RESULTS

Arogenate Dehydratase. Organisms that form L-arogenate as a precursor of L-phenylalanine possess arogenate dehydratase. Arogenate dehydratase may either exist in the absence of prephenate dehydratase as in *Euglena gracilis* (Byng et al., 1981) or coexist with prephenate dehydratase as part of a dual-pathway network leading to L-phenylalanine, as in several pseudomonad groupings (Byng et al., 1983). Arogenate dehydratase was prepared for study from *P. diminuta* and *P. aeruginosa*, these species exemplifying the former and latter categories of pathway patterns, respectively.

Arogenate dehydratase from *P. diminuta* is specific for L-arogenate and will not utilize prephenate in the dehydratase reaction (Whitaker et al., 1981). Enzyme activity in crude extracts [specific acivity = 1.2 nmol min^{-1} (mg of protein)$^{-1}$] was stable during storage at −80 °C for at least 1 month. Arogenate dehydratase form *P. diminuta* ATCC 13184 was partially purified by DEAE-cellulose chromatography as described under Materials and Methods. A single sharp peak of stable activity (Figure 2) was recovered from the salt gradient. Fractions 150–180 were combined to yield a preparation having a specific activity of 6.3 nmol min^{-1} (mg of protein)$^{-1}$. Two assay routines were carried out in parallel with the partially purified enzyme in order to compare the oxidation–fluorescent assay (Shapiro et al., 1981) with the radioactive assay described under Materials and Methods. Both assays yielded similar data (within 5%) when activity was followed as a function of time or as a function of enzyme concentration. Activity was a proportional function of enzyme concentration below 40 μg of partially purified enzyme (contained within a final reaction volume of 200 μL). Under the latter conditions, activity was proportional to reaction time for at least 60 min at 37 °C. The pH optimum for arogenate dehydratase activity from *P. diminuta* ATCC 13184 was defined within the range between pH 7.0 and pH 8.5, activity determinations being made at 0.2 pH unit intervals. A nearly flat pH curve was obtained between pH 7.0 and pH 8.0, with activity levels falling sharply above pH 8.2. Assay of activity below pH 7.0 was not feasible because of the vulnerability of L-arogenate to aromatization at mildly acidic pH (Zamir et al., 1983b). Figure 2 (inset) shows a double-reciprocal plot of substrate saturation data obtained with the partially purified arogenate dehydratase from *P. diminuta* ATCC 13184. A $K_{m,app}$ value of 0.63 mM was obtained from this plot.

Table I: Inhibition of Arogenate Dehydratase from *P. diminuta* by L-Phenylalanine

substrate concentration	inhibitor concentration[a]	specific activity[b]
0.136 mM L-arogenate	0	4.9
	0.05	3.3
	0.10	1.8
	0.25	0
	0.50	0
0.68 mM L-arogenate	0	11.7
	0.05	10.3
	0.10	8.3
	0.25	6.2
	0.50	1.4

[a] Final concentration in millimolar L-phenylalanine added as inhibitor. [b] Expressed as nanomoles of L-phenylalanine formed per minute per milligram of protein.

Sensitivities of Arogenate Dehydratase Enzymes to Inhibition by L-Phenylalanine. The activity of arogenate dehydratase from both *P. aeruginosa* and *P. diminuta* ATCC 11568 was assayed in the presence and the absence of L-phenylalanine by using the HPLC technique described under Materials and Methods. Table I shows that the enzyme from *P. diminuta* ATCC 11568 was strongly inhibited by L-phenylalanine. At 0.136 mM L-arogenate, 100% inhibition was obtained by 0.25 mM L-phenylalanine. The analogues of L-phenylalanine, *p*-fluorophenylalanine, and β-2-thienylalanine were also found to be potent inhibitors of arogenate dehydratase. Inhibition is undoubtedly competitive since the degree of inhibition was significantly diminished at elevated substrate concentration (0.68 mM). The technique used was not sufficiently accurate for obtaining detailed kinetic data, primarily because quantitation was based upon peak heights rather than on peak areas. Variation in sample injections was corrected by inclusion of L-leucine in all injections as an internal standard to which peak heights for L-arogenate and L-phenylalanine were normalized. Quantitation of substrate used or product formed was obtained from standard curves generated through the use of authentic L-arogenate and L-phenylalanine.

In contrast to the *P. diminuta* enzyme, arogenate dehyratase from *P. aeruginosa* was insensitive to inhibition by L-phenylalanine. The three subforms of arogenate dehydratase described by Patel et al. (1977) were separated, and each was found to be insensitive to inhibition by L-phenylalanine. For example, with subform A at 0.068 mM L-arogenate, 10.8 and

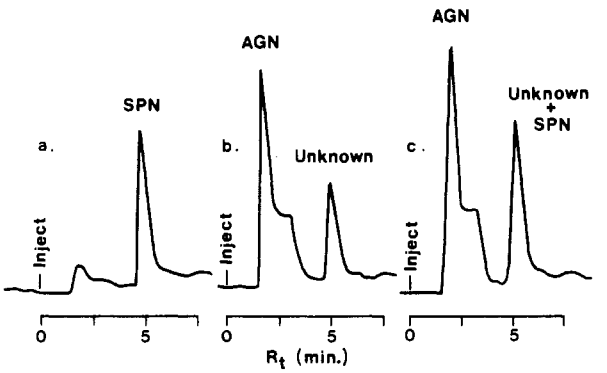

FIGURE 3: Identity of authentic spiro-arogenate (SPN) and "unknown" compound with respect to retention time (R_t) on HPLC. (a) HPLC profile of an authentic standard of spiro-arogenate. (b) HPLC profile of an L-arogenate (AGN) solution that had been boiled 50 min at pH 12 and then diluted 2-fold with distilled water. (c) HPLC profile of a mixture of (a) and (b) in equal proportions. Column: Altex Ultrasphere ODS 1.

11.0 nmol of L-arogenate were utilized from a 200-μL reaction mixture in 60 min at 37 °C in the absence and presence of 0.5 mM L-phenylalanine, respectively. In the same samples the corresponding amounts of L-phenylalanine formed were determined to be 12.6 and 13.7 nmol.

Identification of Product from Thermal Treatment of L-*Arogenate.* When L-arogenate preparations were heated at 100 °C in the pH range 7.5–12, an unknown product was recognized by using HPLC analysis. Panel a of Figure 3 shows the HPLC profile of authentic spiro-arogenate, exhibiting an identical retention time to the unknown compound formed from L-arogenate at 100 °C as shown in panel b. When an L-arogenate sample that had been held at 100 °C under the conditions shown in Figure 3b was spiked with authentic spiro-arogenate, the resulting composite peak (R_t = 5 min) showed no new shoulders (Figure 3c). When L-arogenate solutions were adjusted to about pH 12 by addition of 25 μL of 1 N NaOH/mL of L-arogenate solution, thermal formation of spiro-arogenate, increased by about 50%. At pH 14, on the other hand, very little spiro-arogenate was produced at 100 °C. Thus, an optimal pH for thermal conversion of L-arogenate to spiro-arogenate exists between pH 7.5 and pH 12.0.

If the new product were indeed spiro-arogenate, then acidification of the heated preparation prior to HPLC injection ought to result in an altered HPLC profile whereby the peaks corresponding to L-arogenate and the unknown compound disappear and a new peak at the R_t position of authentic L-phenylalanine forms in an amount equivalent to the sum of L-arogenate and spiro-arogenate present before acidification. This expectation was readily realized as shown in Figure 4.

We considered the possibility that the new compound might originate from a compound contaminating L-arogenate preparations. If so, then acid-catalyzed conversion of L-arogenate to L-phenylalanine prior to boiling at pH 12 would most likely not influence the formation of the new compound. However, such acidification prior to boiling at pH 12 completely abolished formation of the new compound.

Further evidence that the unknown compound was spiro-arogenate was obtained by demonstration of its conversion to L-phenylalanine at acidic pH. The portion of HPLC effluent containing only the new compound was acidified with 1 N HCl and heated at 37 °C for 30 min. After neutralization with 1 N NaOH, an aliquot was treated with OPA and subjected to HPLC analysis. The OPA derivative of authentic L-phenylalanine and the OPA derivative of the acid-treated unknown eluted identically at R_t = 13 min. When OPA-

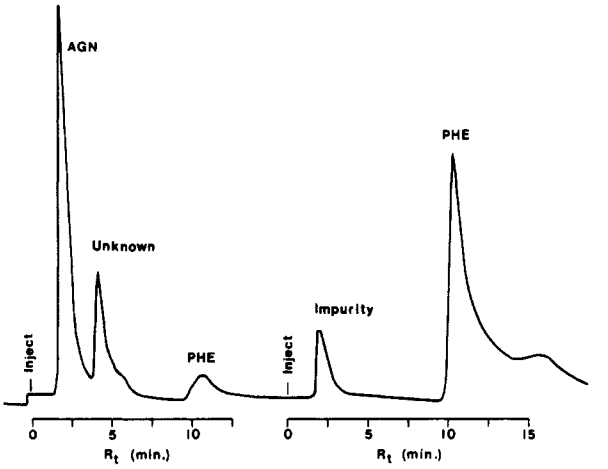

FIGURE 4: Conversion of "unknown" compound to L-phenylalanine (PHE). HPLC profiles are shown for an L-arogenate (AGN) solution boiled 30 min at pH 12 (left) and for an identically treated solution after acidification with 1 N HCl. Column: Altex Ultrasphere ODS 2.

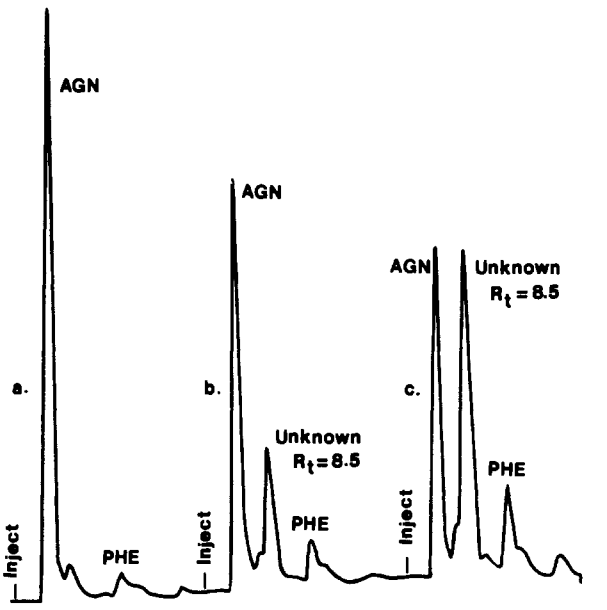

FIGURE 5: Time courses of "unknown" production from L-arogenate (AGN). (a) HPLC profile of an unheated solution of AGN at pH 12. (b) HPLC profile of an AGN solution after boiling 30 min at pH 12. (c) HPLC profile of an AGN solution after boiling for 70 min at pH 12. Column: Altex Ultrasphere ODS 2.

phenylalanine and the derivative of the acid-treated unknown were mixed and then injected into the HPLC, a single sharp peak at R_t = 13 was obtained.

Ambient Conditions Favoring Conversion of L-*Arogenate to Spiro-arogenate.* At temperatures within the range of 37–50 °C, no detectable spiro-arogenate was formed from L-arogenate at pH 7.5 or pH 12.0, even after 22 h. One preparation of L-arogenate was kept at room temperature (pH 7.5) for 6 days; no spiro-arogenate was detected by the sequential acidification and ^{3}H-labeled dansylation method (Zamir et al., 1983b,c). Continued heating of L-arogenate at 100 °C within the range of pH 7.5–12.0 resulted in a steady time course of spiro-arogenate formation and L-arogenate depletion. Figure 5 shows results obtained from samples (pH 12) heated at 100 °C for 30 and 70 min. The unheated sample (left panel of Figure 5) contained a low level of phenylalanine that characteristically contaminates L-arogenate preparations. At pH 12 a progressive conversion of L-arogenate to spiro-arogenate was seen as a function of incubation time at 100

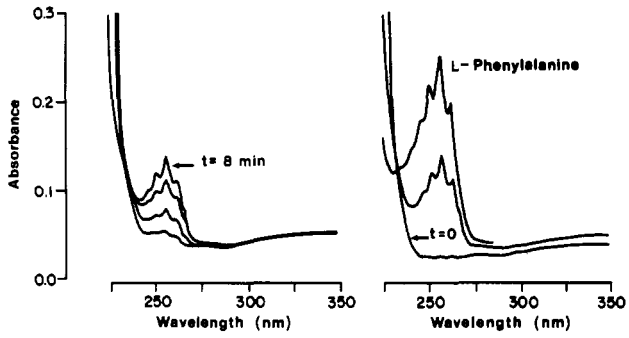

FIGURE 6: Synonymy of UV spectrum of "unknown" with that of L-phenylalanine after acidification. An HPLC eluate containing "unknown" compound obtained from heated AGN was analyzed 0 (bottom right), 2, 4, 6, 8 (left), and 10 (middle right) min after acidification with 1 N HCl. The UV spectrum of authentic L-phenylalanine is shown at the upper right.

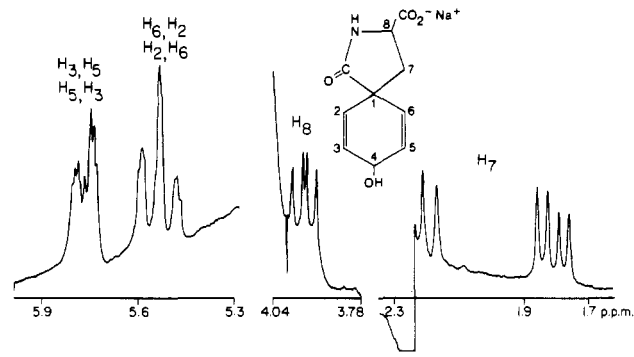

FIGURE 7: Identification of unknown compound as spiro-arogenate by proton NMR. The 200-MHz ^1H NMR of the unknown compound in D_2O that was produced by heating 2 mg of L-arogenate in phosphate buffer is shown. The assignments given for the hydrogen atoms are in accordance with the structure shown for spiro-arogenate. The data were obtained by use of a Varian XL-200 instrument.

°C. After about 70 min at 100 °C spiro-arogenate reached a concentration of about 0.3 mM (40% conversion), as determined by comparison with spiro-arogenate standards. After about 2 h at 100 °C when a conversion of nearly 50% was obtained, the rate of spiro-arogenate formation slowed substantially, perhaps as equilibrium was approached.

TLC Identification of HPLC-Purified Spiro-arogenate. Ten microliters of the HPLC effluent (from a preparative ODS column containing the putative spiro-arogenate) was collected and placed in a quartz cuvette. An ultraviolet spectrum was obtained on a Cary 118 UV–vis spectrophotometer. The spectrum showed only the end absorption consistent with expectations for authentic spiro-arogenate. Fifty microliters of 1 N HCl was then added to the cuvette at room temperature, after which a spectrum was taken every 2 min for 10 min. Figure 6 shows that the spectra which developed were identical with that of authentic L-phenylalanine.

Mass Spectra. The HPLC effluent corresponding to the putative spiro-arogenate peak was collected from several injections of an L-arogenate solution heated at 100 °C at pH 7.5 for 2.5 h. These effluents were pooled and lyophilized. The residue containing spiro-arogenate and phosphate salts was treated with a 1:1 mixture of dimethylformamide (DMF) and bis(trimethylsilyl)trifluoroacetamide. The trimethylsilyl derivative was directly inserted through the inlet source of a Finnegan 3300 mass spectrometer at 70 eV. The mass spectrum obtained of the derivatized spiro-arogenate showed ions at m/e 425, an $n-1$ peak at m/e 424, and a loss of CH_3^+ (m/e 410) in agreement with the spectrum obtained for authentic spiro-arogenate having attached trimethylsilyl groups (Zamir et al., 1983c). Other peaks were also observed in the spectrum that implicated other compounds having relatively higher solubility in DMF compared to sodium spiro-arogenate.

^1H Nuclear Magnetic Resonance of the Unknown Compound. The 200-MHz ^1H NMR spectra of the unknown obtained from the heating of L-arogenate confirmed its structure as spiro-arogenate (Figure 7). The ^1H NMR signals of this compound in D_2O were identical with the published ^1H NMR of spiro-arogenate (Zamir et al., 1983c). The HOD peak appears at 4.588 ppm. The proton at position 7 shows as an AB(X) pattern with the following values: For 7A, ν = 364.27 (J_{AB} = −13.31) and δ = 1.821 ($J_{7A\text{-}8}$ = −6.23); for 7B, ν = 445.35 and δ = 2.226 ($J_{7B\text{-}8}$ = 8.55). The proton at H-8 is a doublet of doublets (DD) with the values ν = 790.43 and δ = 3.951 ppm. The olefinic protons show the following pattern: H_6 or H_2, $\delta \sim 5.514$ ($J_{cis} \sim 10.62$); H_2 or H_6, $\delta \sim 5.567$ ($J_{2\text{-}4} = J_{26} = 1.3$); H_5 or H_3, $\delta \sim 5.774$ ($J_{cis} \sim 10.0$); H_3 or H_5, δ = 5.774 ($J_{3\text{-}4} = J_{5\text{-}4} = 1.8$). The proton at H_4 is a multiplet which is obscured by a strong HOD peak.

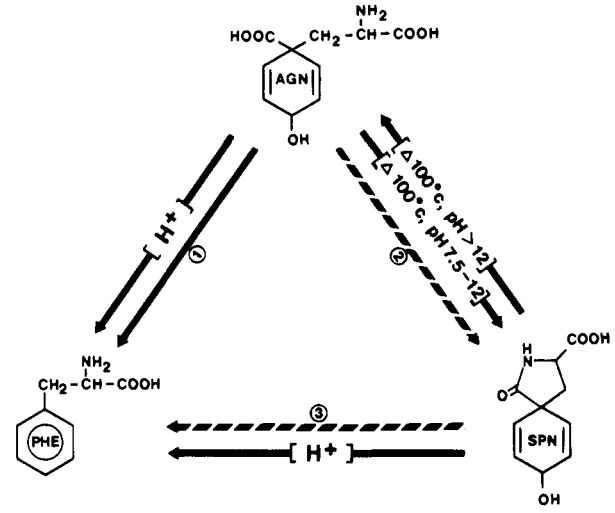

FIGURE 8: Enzymatic and nonenzymatic interconversions between L-arogenate (AGN), spiro-arogenate (SPN), and L-phenylalanine (PHE). Enzymatic reactions are indicated by number. Enzyme 1 (arogenate dehydratase) is an established protein catalyst, whereas the spirase (reaction 2) and spiro-arogenate dehydratase (reaction 3) are hypothetical.

Discussion

Figure 8 indicates the enzymatic and nonenzymatic conversions that are known to intervene between L-arogenate, spiro-arogenate, and L-phenylalanine. Arogenate dehydratase is now well established (Byng et al., 1982). Two types of the latter enzyme have thus far been noted. In organisms like *Euglena gracilis* (Byng et al., 1981) and *Pseudomonas diminuta* (Whitaker et al., 1981) where L-arogenate is apparently an obligatory precursor of L-phenylalanine, the enzyme does not accept prephenate as a substrate. In organisms like *P. aeruginosa* (Patel et al., 1977; Whitaker et al., 1981) where dual enzyme routes to L-phenylalanine coexist, arogenate dehydratase will accept either prephenate or L-arogenate as substrate. Spiro-arogenate has no known biological function, and the existence of a "spirase" enzyme (Zamir et al., 1983c) is presently hypothetical. The ambient conditions demonstrated in this paper for spiro-arogenate formation (e.g., 100 °C) in vitro support the likelihood that its accumulation in vivo would be dependent on the presence of an enzyme catalyst.

Figure 8 shows that both L-arogenate and spiro-arogenate are aromatized to form L-phenylalanine at acidic pH. Spiro-arogenate probably proceeds to L-phenylalanine via an

L-arogenate intermediate. A detailed account of the acid-catalyzed aromatizations of prephenate, L-arogenate, and spiro-arogenate was presented by Zamir et al. (1983b). The lactam ring of spiro-arogenate is cleaved by boiling in strong base to yield L-arogenate. At neutral to mildly alkaline pH, high temperature (100 °C) favors steady conversion of L-arogenate to spiro-arogenate until 50–60% conversion has occurred after about 2 h. The optimal pH for this conversion (somewhere between pH 7.5 and pH 12.0) probably reflects a balance between the effect of higher pH in preventing aromatization that occurs more and more readily as temperature is elevated and the offsetting tendency of progressively higher pH to favor breakage of the lactam ring.

The conversion of L-arogenate to spiro-arogenate is perhaps surprising. We suggest that hydrogen bonding between an amino hydrogen and the carboxylate moiety at C-1 would facilitate dehydration to produce the five-membered lactam ring. The proposed hydrogen bonding is consistent, furthermore, with the demonstration of greater stability of L-arogenate than of prephenate with respect to aromatization (Zamir et al., 1983b).

Arogenate dehydratase is a difficult enzyme to assay. The oxidation–fluorescent assay of Shapiro et al. (1981) is sensitive and relatively inexpensive. However, it requires a variety of controls because of the potential oxidation of organic compounds present (other than L-arogenate) by $KMnO_4$. The presence of other fluorescent compounds can also interfere with the measurement of the fluorescent derivative of L-phenylalanine that is employed in the assay. The radioactive assay described in this paper is more specific and at least twice as sensitive as the oxidation–fluorescent assay. It is more expensive and more tedious, however. We currently use the oxidation–fluorescent assay for preliminary assays and for screening-type procedures, e.g., location of an activity peak in the eluate of a chromatographic column. The radioactive assay is used where precise enzymological data are sought.

Arogenate dehydratase from *P. diminuta* was used to affirm the usefulness of the new radioactive assay. Although the enzyme from this organism has not been studied in comprehensive detail, it currently stands as the best characterized arogenate dehydratase of the class specific for L-arogenate. Its sensitivity to end product inhibition by L-phenylalanine is the first example of a regulated arogenate dehydratase. The HPLC technique employed to demonstrate end product inhibition is tedious because relatively small differences in substrate disappearance or product appearance must be followed against relatively large backgrounds of substrate or product (i.e., added L-phenylalanine). Detailed kinetics of inhibition await the isolation of ^{14}C-labeled L-arogenate so that labeled L-phenylalanine (product) can be discriminated from unlabeled L-phenylalanine (added to the reaction mixture as a feedback inhibitor). The qualitatively effective inhibition by L-phenylalanine demonstrated for the *P. diminuta* enzyme completes a picture of tight overall pathway control for this organism in which each aromatic amino acid participates in regulation at key branchpoint positions (Byng et al., 1983).

The second class of arogenate dehydratase examined was from *P. aeruginosa*. Since this enzyme can also utilize prephenate as substrate, the characterization of this enzyme has often exploited assay of its prephenate dehydratase activity since both substrates probably bind to a common site; i.e., in cases where arogenate dehydratase possesses prephenate dehydratase activity, it is technically easy to measure sensitivity of the latter activity to inhibition by L-phenylalanine. We had previously found that arogenate dehydratase (assayed as prephenate dehydratase) was not sensitive to feedback inhibition by L-phenylalanine (Patel et al., 1977). In this paper we show directly that arogenate dehydratase is indeed insensitive to end product inhibition. This is consistent with the results of Fiske et al. (1983), who showed that a mutant of *P. aeruginosa* deregulated for an early-pathway enzyme excreted L-phenylalanine via the L-arogenate branch which accommodated the overflow of common-pathway intermediates.

Registry No. L-Arogenic acid, 53078-86-7; spiroarogenic acid, 86334-99-8; arogenate dehydratase, 76600-70-9; L-phenylalanine, 63-91-2.

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