Phenylalanine Ammonia-lyase: Enzymic Conversion of 3-(1,4-Cyclohexadienyl)-L-alanine to *trans*-3-(1,4-Cyclohexadienyl)acrylic Acid†

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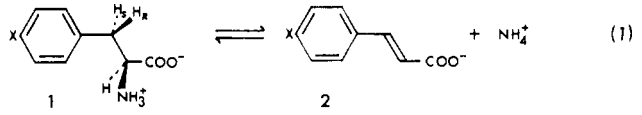
ABSTRACT: The phenylalanine analogue 3-(1,4-cyclohexadienyl)-L-alanine is converted to the hitherto unknown cinnamate analogue *trans*-3-(1,4-cyclohexadienyl)acrylic acid by L-phenylalanine ammonia-lyase (EC 4.3.1.5) from maize, potato, or *Rhodotorula glutinis*. The structure assigned to the product is confirmed by its ^1H nuclear magnetic resonance spectrum and by the chemical synthesis to be described in a subsequent paper. On comparing the above substrate analogue with L-phenylalanine, the K_m was lowered only slightly but k_{cat} was reduced 14–40-fold depending on the source of the enzyme. Because the compounds closely resemble each other in size and hydrophobic properties, this lowering of k_{cat} may be attributed to the electronic effect of replacing the π electrons of the aromatic system by those of a double bond. Correct alignment at the active site appears to depend upon the space-filling properties of the ring system; open chain analogues that retain the γ,δ double bond were found to be inhibitors, not substrates.

When HX is eliminated in enzyme-catalyzed reactions, the β carbon is usually activated by a carboxylate ion, an aromatic system, or an ester or thioester group. To the best of our knowledge no enzymic elimination is known in which the β activation is provided by an isolated double bond. The availability of 3-(1,4-cyclohexadienyl)-L-alanine, **3** (Snow et al., 1968, Ressler, 1972),[1] and L-phenylalanine ammonia-lyase made it possible to inquire whether a double bond can provide sufficient β activation to allow catalysis to take place at a reasonable rate. The enzyme catalyzes the elimination of the (*pro*-3*S*)-H and NH_3 from L-phenylalanine to give *trans*-cinnamate as in eq 1, X = H (review: Hanson & Havir, 1972).



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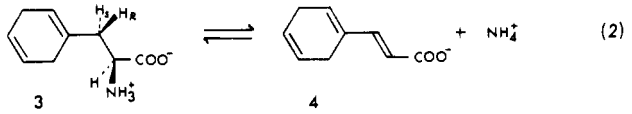
In this paper we show that the reaction of eq 2 does indeed



(2)

occur to yield the hitherto unknown ring dihydro analogue of cinnamate: *trans*-3-(1,4-cyclohexadienyl)acrylate, **4**. The kinetic results are discussed in relation to the biosynthesis of compounds structurally related to sorbic acid and to the mechanism of the elimination reaction. A subsequent paper will describe the chemical synthesis of the dihydro acid **4** and its biological activity (Ressler et al., 1979).

Experimental Section

General. Proton magnetic resonance spectra were obtained on a Varian EM-360 spectrometer. The $CHCl_3$ signal at δ 7.27 was used as an internal reference. Coupling constants were obtained from direct spacing measurements. Infrared spectra using KBr disks were taken on a Perkin-Elmer Model 137 spectrophotometer. Ultraviolet absorption spectra were determined, and all enzyme assays and kinetic measurements were performed, with a Gilford 2400 recording spectrophotometer. Elemental analyses were determined by Micro-Tech Laboratories, Skokie, IL. Radioactivity was assayed with a Searle Analytic Isocap/300 liquid scintillation counter using an ethanol–toluene-based scintillation fluid. Where appropriate, the distribution of radioactivity in electrophoresis papers, etc., was determined by adding strips of the paper to vials containing scintillation fluid.

Paper electrophoretic separations were performed at ~10 °C with a 45-cm flat-plate system (Savant) on Whatman 3 MM paper. With acetate (Na^+) buffer (0.02 M), pH 4.4, or with pyridine–acetate buffer (4 mL + 8 mL per L), pH 4.4, good separations were obtained at 3 h (67 V/cm) and 4 h (44 V/cm), respectively. Typical migration distances for phenylalanine, the dihydro acid, and cinnamic acid were 2, 17, and 32 cm, respectively. With formic acid (2.6 mL/L), pH 2.0, 2 h and 44 V/cm, phenylalanine fully separated from cinnamic acid and its dihydro analogue (e.g., 20 cm compared with 5 cm). Both cinnamic acid and its dihydro analogue could be detected on the dried papers under 254-nm UV light as quenching spots. With acetate buffer the limit of detection was 25 nmol on 3 MM paper and 0.03 nmol on Whatman no. 1 paper. With pyridine–acetate buffer quenching by residual pyridine on the dried paper greatly reduced sensitivity.

Phenylalanine ammonia-lyase from maize and potato was prepared as described (Havir & Hanson, 1970, 1973; Havir et al., 1971) and the enzyme from *Rhodotorula glutinis* was purchased from P-L Biochemicals.

The reference compounds sorbic acid (99+%) and *trans*-cinnamic acid (zone refined, 99.9+%) were purchased from

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[1] Abbreviation and nomenclature used: under the IUPAC–IUB 1974 recommendations (1975), the compound (2*S*)-2-amino-3-(1,4-cyclohexadienyl)propanoic acid may be named 3-(1,4-cyclohexadienyl)-L-alanine or 2,5-dihydro-L-phenylalanine. The latter name leads to the abbreviation L-DiHPhe. The compound (*E*)-3-(1,4-cyclohexadienyl)-2-propenoic acid which could be named as *trans*-3-(1,4-cyclohexadienyl)acrylic acid or as a ring 2,5-dihydro derivative of cinnamic acid will be referred to as the "dihydro acid" for convenience. (*E*,*E*)-Hexa-2,4-dienoic acid has the trivial name sorbic acid. As with the cinnamic acids, the trivial name may be used in conjunction with configurational prefixes, e.g., of the four stereoisomers, *trans*,*cis*-sorbic acid implies (2*E*,4*Z*)-hexa-2,4-dienoic acid. The choice of convenient names for the above compounds is complicated by the fact that acrylic acid, cinnamic acid, and sorbic acid are not root names for indexing in Chemical Abstracts. Other abbreviations: LC, high pressure liquid chromatography; Boc, *tert*-butoxycarbonyl.

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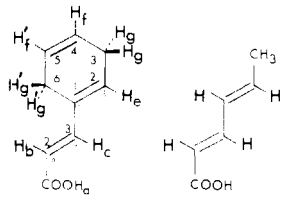
Aldrich. Cyclohexyl-L-alanine was a gift of Dr. Joseph Fruton. DL-Allylglycine and L-β-cyanoalanine were purchased from ICN Pharmaceuticals.

3-(1,4-Cyclohexadienyl)-L-alanine (**3**). Unlabeled L-DiHPhe was prepared from L-phenylalanine by a Birch reduction using large molar excesses of Na and methanol in NH_3 at −40 °C (Snow et al., 1968). The crystalline product used in the preparative experiments when assayed by an automatic amino acid analyzer was found to contain <3% L-phenylalanine. U-^{14}C-labeled material (11.6 μmol, 4.31 Ci/mol) was prepared from L-[U-^{14}C]phenylalanine (0.108 μmol, 460 Ci/mol, from Schwarz/Mann) and unlabeled L-phenylalanine (11.5 μmol) by reduction under conditions shown in parallel experiments without isotope to yield L-DiHPhe containing <1% L-phenylalanine.

Enzymic Preparation of trans-(1,4-Cyclohexadienyl)acrylic Acid (**4**). The pH 8.7 borate (Na^+) buffers for the reaction and for dialysis were degassed under reduced pressure and then saturated with N_2. The reaction was carried out in a standard flask closed with a serum stopper. The space above the liquid was flushed with N_2 by means of a pair of hypodermic needles. The reaction was monitored by withdrawing samples (0.5 mL) with a syringe, diluting these with water (1.5 mL), and determining the absorbancy of the product (ϵ_{290} = 2400; ϵ_{295} = 1200). Recently prepared L-DiHPhe (3 mmol, 502 mg) in water (12 mL) at pH 5 was stored at −10 °C as three separate samples. Phenylalanine ammonia-lyase from potatoes (50 units, 0.55 unit/mg of protein, 100 mL) was dialyzed under N_2 against 0.04 M buffer. The first portion of L-DiHPhe (1 mmol) was treated with the dialyzed enzyme in 0.04 M buffer (1 L) at 35 °C and the reaction was monitored for 4 days. The enzyme was then precipitated at 0 °C with $(NH_4)_2SO_4$ (459 g; 65% saturation), collected by centrifugation, dialyzed against buffer, and used to treat the second portion of L-DiHPhe. The third portion was treated similarly. Essentially all of the protein and 75% of the enzyme activity added were recovered in each cycle. Immediately after the centrifugation step, each supernatant $(NH_4)_2SO_4$ solution was acidified to pH 2 with concentrated HCl (5 mL) and extracted with ether (4 × 90 mL). The extract was washed with water (50 mL), dried over Na_2SO_4, concentrated by distillation, and taken to dryness under N_2. The yield (~85%) of **4** was calculated from the absorbancy of a sample taken from the ether solution diluted 100-fold with 0.02 M buffer (ϵ_{263} = 15800). In each case the yield agreed with the weight of colorless plates obtained (e.g., 126 mg, mp 152–155 °C). For analysis 89 mg was recrystallized twice from ethanol to furnish 43 mg, mp 155.5–157.5 °C. Anal. Calcd for $C_9H_{10}O_2$: C, 72.0; H, 6.71. Found: C, 72.1, H, 6.75. IR: 3100–2870 (COOH), 1695 (=CHCO), 1620 (COO^-, C=C—C=C), 1425, 1335, 1225, 980, 877, and 700 cm^{-1}; other physical data are given below. The compound resembled cinnamic acid in both its crystalline form and characteristic odor. A low intensity signal at δ 7.5 in the ^{1}H NMR spectrum of this material is attributable to a phenyl ring and indicates the presence of cinnamic acid (<4%) as a contaminant. The peak was not observed in the spectrum of synthetic material purified by LC (Ressler et al., 1979). The same material examined over a year later by LC contained ~6% cinnamic acid. Although enzyme from potatoes was employed in the above preparation, commercially available *R. glutinis* enzyme is expected to behave similarly. The use of immobilized enzyme or enzyme recovery by ultrafiltration should be considered.

^{1}H NMR Spectrum. The 60-MHz spectrum of the product is in keeping with its postulated structure as the dihydro acid **4**. In the equivalent planar structure **5**, pairs of enantiotopic



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protons, which must give identical signals, are designated in the same way. All other protons are constitutionally heterotropic (Hirschmann & Hanson, 1971) and, in principle, give distinct signals.

The pairs $2H_g$ and $2H_g'$ are in constitutionally similar environments and should have similar chemical shifts. The observed broad 4H signal at δ 2.84 is thus attributable to these protons (cf. the broad 4H singlet at δ 2.61 observed for DiHPhe: Snow et al., 1968). Because of the 60° torsion angle between the bonds to H_g or H_g' and the protons on adjacent carbons, the coupling constants to these must be small (~2 Hz: Williamson & Johnson, 1961); thus the signals for H_g, H_g', H_f, H_f', and H_e are broadened rather than split. As there is no significant difference in chemical shift between H_g and H_g' homoallylic coupling is not observed (see Rabideau, 1978).

The olefinic protons H_f and H_f' are also in constitutionally similar environments and their signals should be close to the A_2 limiting case of an AB system and give a single peak. The observed peak at ~5.82 is assigned, therefore, to $H_f + H_f'$ (cf. 2H peak at ~5.69 for DiHPhe).

The spectra of *trans*-cinnamic acid, **2** (X = H), and *trans,trans*-sorbic acid, **6**, provide grounds for assigning signals to H_b, H_c, and H_e and establishing the configuration of the double bond. In cinnamic acid the α and β olefinic protons are interpreted as an AB system with a significant chemical shift between the two doublets centered at δ 6.48 and 7.84. The doublets of the product at δ 5.78 and 7.47 are assigned by analogy. The signals are further upfield than for cinnamic acid reflecting increased deshielding by the aromatic system relative to the doublet bond system (Jackman, 1959). The apparent absence of an H_b doublet centered at δ 7.84 is consistent with the low content of cinnamic acid in the sample.

The observed coupling constant J_{bc} was 15.8 ± 0.2 Hz. This is in the range characteristic for proton–proton spin coupling across a trans double bond (Williams & Flemming, 1966). J_{bc} for *trans*- and *cis*-cinnamic acids is 15.8 and 12.3 Hz, respectively (Baden et al., 1966). In methyl *trans,trans*-sorbate, the coupling constants for J_{ab} and J_{bc} are 15.8 and 15.1 Hz, whereas in methyl 2-*cis*,4-*trans*-sorbate they are 11.6 and 15.7 Hz, respectively (Elvidge & Ralph, 1966). The close correspondence in the chemical shifts observed for the product and for the α and β protons of *trans,trans*-sorbic acid (δ 5.77 and 7.40) also indicate that H_b and H_c are in a trans orientation. This leaves the remaining signal at δ 6.26 to be assigned to the olefinic proton H_e.

The assignment of H_e may also be justified by reference to the corresponding proton in DiHPhe and a variety of its derivatives. These compounds gave an H_e signal less downfield than the other two olefinic protons corresponding to H_f and H_f', the three being generally within the range of δ 5.60–5.95. [The list includes carbobenzoxy and Boc *p*-nitrophenyl esters of DiHPhe in $CDCl_3$, Boc-L-DiHPhe in MeOD (Nagarajan et al., 1973), acetyl DiHPhe in Me_2SO, and DiHPhe in D_2O (Snow et al., 1968)]. A pronounced downfield shift of signal in going from DiHPhe to the product is consistent with the conjugation of H_e to an α,β-unsaturated system as in **5**.

Table I: UV Absorbtion Spectra of *trans*-3-(1,4-Cyclohexadienyl)acrylic Acid, 4, and Reference Compounds[a]

		dihydro acid (4)	*trans,trans*-sorbic acid (6)	*trans*-cinnamic acid (2)	structural λ_{max} shift (4 minus 6)
	In Organic Solvents				
n-hexane	λ_{max}, nm	267[b]	256	274	11
	ϵ_{max}	(12 800)	(17 400)	(15 500)	
ethanol, 95%	λ_{max}, nm	265[c]	254[d]	272[e]	11
	ϵ_{max}	(16 000)	(22 300)	(19 600)	
	In Buffers				
pH 2.3[f] (all COOH)	λ_{max}, nm	272	263	278	9
	ϵ_{max}	(15 700)	(24 400)	(18 300)	
pH 7.0[f,g] (all COO^-)	λ_{max}, nm	262	254	268	8
	ϵ_{max}	(15 800)	(23 400)	(17 800)	
isosbestic point	λ_{IBP}, nm	266.5	257.5	272.0	9
	ϵ_{IBP}	(15 100)	(22 800)	(17 500)	
ionization λ_{max} shift (COOH minus COO^-)		10	9	10	

[a] The spectral and spectrophotometric titration data collected for the following variants of the cinnamic acid structure fall into the pattern shown in this table and Figure 1. λ_{max}, nm (ϵ_{max}) etc. as in this table. Sequence: COOH, COO^-, isosbestic point, ionization λ_{max} shift (COOH minus COO^-), pK_a. *trans-p*-Methylcinnamic acid: 287 (20 200), 276 (19 800), 281 (19 300), 11, 4.22. *trans-m*-Methylcinnamic acid: 280 (19 400), 271 (19 200), 274 (18 300), 9, 4.07. *trans-O*-Methylcinnamic acid: 280 (15 900), 270 (15 400), 274 (14 900), 10, 4.01. *trans*-3-(2-Thienyl)acrylic acid: 293 (16 000), 302 (14 900), 303.5 (14 600), 10, 4.18. The methylcinnamic acids and cinnamic acids all showed absorbancy maxima in the 220-nm region and a trough in the 230–238-nm region. The maxima were not observed for *trans*-3-(2-thienyl)acrylic acid, the dihydro acid, or *trans,trans*-sorbic acid. [b] No fine structure revealed. [c] λ_{min}, 210 nm (3500). [d] Lit.(Allan et al., 1955; Eisner et al., 1953). Sorbic acids, λ_{max}, nm (ϵ_{max}): *trans,trans*, 254 (26 000); *trans,cis*, 260 (22 500); *cis,trans*, 257 (21 000); *cis,cis*, 259 (20 000). Ionization λ_{max} shift (COOH minus COO^-): *trans,trans*, 9.5 nm; *cis,cis*, 13 nm. λ_{min}, all ~220 nm. [e] Lit. (Scott, 1964). Cinnamic acids: *trans*, 273 (21 000); *cis*, 264 (9500). [f] McIlvaine's buffer systems (Whiting, 1966) as Figure 1. [g] Same values in borate (Na^+) buffer, pH 8.7.

Steric interactions play an important role in determining the conformational preferences about nominally single bonds in conjugated systems (Honig et al., 1975); thus in solution the β–γ bond in **5** could be *s*-trans, as shown, or *s*-cis, or an equilibrium mixture of both.

UV Spectra. The single peak near 265 nm in the spectrum of the dihydro acid (Table I) is a typical electron-transfer band, oxygen being the electron sink (Scott, 1964). The finding that **4** absorbs at a longer wavelength than *trans,trans*-sorbic acid is attributable to alkyl substitution and bridge formation. Alkylation in the γ position alone could account for a 9-nm bathochromic shift (less than for the methylation of a conjugated carbonyl system but more than for the methylation of a diene: Nielson, 1957; Scott, 1964, p 76). By the same reasoning one should be able to deduce the UV spectrum of 2,5-dihydrobenzoic acid from that of (*E*)-2-methyl-2-butenoic acid (λ_{max} 213 nm; ε 12 500). However, the former compound has an abnormal spectrum (λ_{max} 236 nm; ε 2100; Jones et al., 1956; Nielson, 1957; Scott, 1964). If 2,5-dihydrobenzoic acid exists in enantiomeric boat conformations so that the π electrons of the bridge double bond interact with the excited state of the acrylic acid system (Kosower et al., 1961), then the higher vinylogue **4** must be planar or near planar.

pK_a Determinations and Symmetry Fit of Titration Data. Figure 1 compares the spectrophotometric titration curves for the dihydro acid and the reference compounds. As expected, the pK_a is similar to that of *trans,trans*-sorbic acid. The weaker acidity of the dihydro acid relative to cinnamic acid is confirmed by the electrophoretic and chromatographic results discussed below.

The following novel approach was employed to fit the spectrophotometric data to a theoretical titration curve. Absorbancy data collected at a particular wavelength and expressed relative to the absorbancy at the isosbestic point were plotted as a function of pH. Replicate data points were traced on a second sheet of paper and the superimposed graphs examined on a light table with one sheet rotated through 180°. The graphs were adjusted until both sets of points were compactly distributed about a smooth symmetrical curve. The

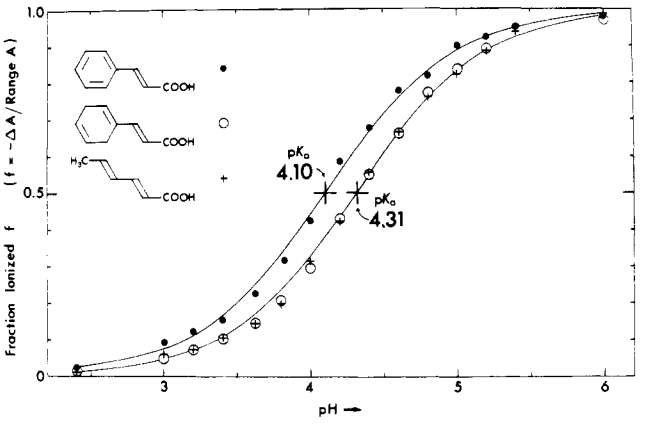


FIGURE 1: Spectrophotometric titrations of *trans*-3-(1,4-cyclohexadienyl)acrylic acid, **4**, *trans,trans*-sorbic acid, **6**, and *trans*-cinnamic acid, **2**. The curves shown are theoretical titration curves defined by $f = 1/(1 + [H^+]/K_a)$ and fitted to the data as described under Experimental Section. UV-absorbancy measurements were made for identical amounts of a given compound in a series of 0.01 M McIlvaine buffer solutions (Na_2HPO_3 and citric acid; Whiting, 1966). Absorbancies were measured at the isosbestic point (Table I) and at wavelengths selected to provide ranges of absorbancy values between 0.2 and 0.4 (**4**, at 280 and 290 nm; **6**, at 275, 280, and 285 nm; and **7**, at 290 and 300 nm). These readings were then expressed as fractions of the isosbestic point readings in order to eliminate the effect of pipetting errors.

inflection point, a center of twofold symmetry, gave the pK_a of the acid. The absorbancy at this point was subtracted from the curve absorbancy values at −0.5, −0.6, −0.7, etc. to −1.2 pH units from the estimated pK_a. Factors were calculated relating these values to the theoretical titration curve of unit range. The average factor was then used to either calculate a theoretical curve through the data points or convert the points to the titration range of unity (Figure 1). A check on the procedure was obtained by making independent observations at three different wavelengths.

Separation Methods. Cinnamic acid is the only probable contaminant of the dihydro acid formed enzymically and its separation from the synthetic compound is required. Sepa-

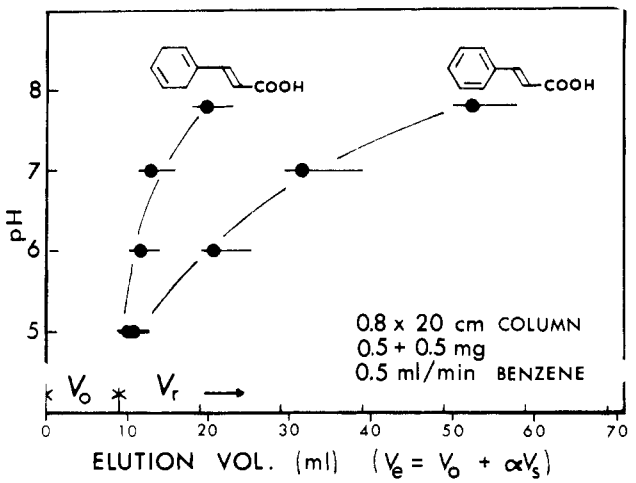


FIGURE 2: Separations on buffered silica gel columns. The dots represent the peak positions and the lines give the width of the peak at half the height ($d_{1/2}$). The void volume was determined with citrate buffer, pH 2 in the stationary phase. Other buffers were citrate–phosphate or phosphate (0.5 M). The buffer (5 mL) was added to 10 g of SilicAR CC-4, 100–200 mesh (Mallinckrodt), and mixed before pouring the column as a slurry in benzene. Elution volumes were slightly less when water-washed benzene was used as the eluant. The number of theoretical plates was calculated to be 110 and 180 for the first and second peaks, respectively [$= 5.45(V_r/d_{1/2})$].

ration can be achieved by zone electrophoresis on paper, by partition chromatography on buffered silica gel columns, and also by LC (Ressler et al., 1979).

The electrophoretic mobilities for the fully ionized acids should be very similar; thus their relative distances of migration should be predictable from their relative pK_a values. As expected, cinnamate migrates faster than the dihydro acid. At pH 4.4 with acetate (Na^+) or pyridine–acetate buffer, the observed ratios ${}^Cd/{}^Dd$ were 1.3 to 1.4 compared with 1.2 calculated from the equation ${}^Cd/{}^Dd = (1 + [H^+]/{}^DK_a)/(1 + [H^+]/{}^CK_a)$, where C and D stand for the cinnamic and dihydro acids, respectively. The limit ratio at low pH is ${}^CK_a/{}^DK_a = 1.6$. These calculations ignore effects of ionic strength and temperature on pH and pK_a values.

Satisfactory analytical separations of the two acids were achieved on silica gel columns buffered with phosphate in the range pH 6–8 using benzene as the mobile phase (Figure 2). As the pH becomes more alkaline, the acids are more ionized and thus spend less of their time in the mobile phase. Load effects appear to cause the system to depart from ideal behavior. If α_a is the partition coefficient of a free acid, $\alpha = \alpha_a(1 + K_a/[H^+])$. The retardation volume for the acid V_r is αV_s where V_s is the volume of the stationary phase. If the pH is 1.5 pH units or more greater than the pK_a, then approximately $V_r = \alpha_a V_s K_a/[H^+]$. The increase in V_r should be, therefore, about tenfold for 1 pH unit, whereas a twofold increase was observed.

For two acids, ${}^CV_r/{}^DV_r = ({}^C\alpha_a/{}^D\alpha_a)({}^CK_a/{}^DK_a)$. If ${}^C\alpha_a = {}^D\alpha_a$, then using the pK_a values of Figure 1 and neglecting ionic strength and temperature effects, ${}^CV_r/{}^DV_r = 1.6$. This implies that the weaker acid (the dihydro acid) is eluted first, as is observed. A series of five columns were run at pH 7 with the load decreasing from 0.5 mg of each acid to 0.01 mg. ${}^CV_r/{}^DV_r$ decreased from 5.5 to 2.0 and the extrapolated value to zero load was about 1.8 instead of 1.6. This suggests that ${}^C\alpha_a/{}^D\alpha_a$ is greater than unity and, therefore, that the dihydro acid is slightly more hydrophobic than cinammic acid. In agreement with this, cinnamate was eluted first on reversed phase LC.

The above load effect complicates the scale-up of the column for preparative use. A 50-fold larger column (5 × 25 cm) comfortably separated 20 mg of each acid. To achieve dynamic similarity (Charm & Matteo, 1971, p 518), a low flow rate (3.1 mL/min) had to be used so that elution of the dihydro acid took 8 h.

(2S,E)-2-Amino-4-methylhex-4-enoic Acid. The sample of this natural product (kindly provided by Dr. Leslie Fowden: Fowden, 1974; Fowden & Smith, 1968) also contained leucine (8%), isoleucine (2%), and a trace of phenylalanine. A portion of the sample (200 μmol) was treated with phenylalanine ammonia-lyase from maize (0.7 U) in 8 mM borate (Na^+) buffer, pH 8.7, 25 mL, for 18 h. The remaining amino acids were recovered by procedures that included dialysis and chromatography on Dowex 50-8W. The treatment was repeated on a portion of the recovered material. Amino acid concentrations were assayed with ninhydrin (Moore, 1968). Kinetic studies with phenylalanine ammonia-lyase showed that, for a given amino acid concentration, initial velocities were reduced by successive treatments about fivefold and then greater than tenfold to the point where no significant activity was observed. Amino acid analyses showed that the L-phenylalanine content had been reduced to the limits of detectability by the enzyme treatments. Leucine and isoleucine appeared to be unaffected by the enzyme.

Inhibition Studies. The above unsaturated amino acid and other acids structurally related to L-DiHPhe and L-phenylalanine have been tested as substrates and inhibitors of the enzyme (see Results). Because a uniform basis of comparison was needed and because certain of the compounds were only available in small quantities, the inhibition studies were carried out at low inhibitor and substrate concentrations. Phenylalanine ammonia-lyase, thought to be a two-protomer enzyme, shows significant negative cooperativity between its subunits (Havir & Hanson, 1968; Hanson & Havir, 1978; Nari et al., 1974; Ricard et al., 1972). The inhibition constants determined at low substrate concentrations, therefore, reflect binding to one of two alternative sites. At a given substrate concentration, values of the initial velocity in the presence and absence of a given inhibitor concentration were determined (v_I and v_S) and the function $A = [I]v_I/(v_S - v_I)$ was calculated. Assuming that only one molecule of substrate or inhibitor binds, $A = K_I(1 + [S])/K_m$. (K_I is the dissociation constant for the competitive binding of the inhibitor to either site expressed in terms of the whole enzyme.) With D-phenylalanine as the inhibitor, values of A were plotted against [S] to give K_I and K_m. The values of [S] used were less than twice the calculated K_m of 0.5 mM. More limited experiments were then carried out with the other inhibitors at low substrate concentrations (0.025, 0.05, and 0.10 mM) and a small correction was applied to the A values on the basis of the calculated K_m to give K_I.

The maize enzyme used in the experiments described had lost essentially all of its cooperativity with respect to phenylalanine, but cooperativity with respect to the inhibitors cannot be ruled out. It is not clear at this time what generalized subunit model is appropriate for describing inhibition phenomena. The above method, based on Nari et al. (1974) and Hanson & Havir (1978), yields K_I values for competitive inhibition which can be used for comparison purposes pending a better understanding of the enzyme.

The inhibition of the enzyme by the dihydro acid was studied by an alternative procedure. L-[U-^{14}C]Phenylalanine (1.05 mM, 1 μCi/mol) in 10 mM borate (Na^+) buffer, pH 8.7, was treated with enzyme (from maize, 19 mU; potato, 11 mU; or *R. glutinis*, 22 mU) in the presence of dihydro acid (4.3 mM or 8.5 mM) or cinnamate (10 mM); final volume was 100 μL. Samples withdrawn at zero time and at a series of 20-min

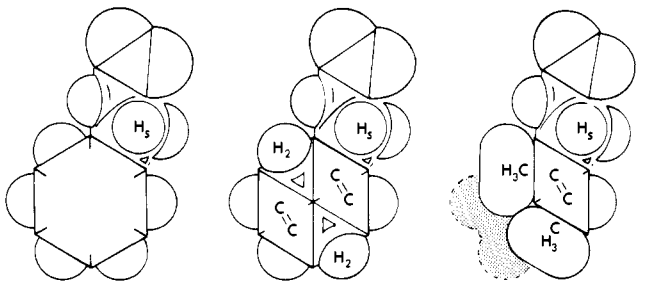


FIGURE 3: Drawings from CPK models, (A) L-phenylalanine (**1**); (B) 3-(1,4-cyclohexadienyl)-L-alanine (**3**); and (C) (2*S*,4*E*)-2-amino-4-methyl-4-hexenoic acid (**7**). The shaded area indicates the difference between models C and A. The models are oriented with the bonds to be broken in the elimination reaction perpendicular to the plane of the paper: C^{α}–N below and C^{β}–H_S above [see Hanson & Rose (1975), Figure 5].

intervals were applied to ChromAR sheets (Mallinckrodt), and the amount of [U-^{14}C]cinnamate formed was determined after chromatographic separation as described previously (Havir et al., 1971).

Results

Enzymic Preparation. In the preparation described in the Experimental Section, phenylalanine ammonia-lyase (50 units/L) converted L-DiHPhe (3, mM) to the dihydro acid in 85% yield during 4 days at pH 8.7 and 30 °C. The conditions for this preparation were established using L-[U-^{14}C]DiHPhe as a substrate and following the reaction both spectrophotometrically at 295 nm and by electrophoresis of samples at pH 4.4. The percentage yield increased as the concentration of substrate decreased and the concentration used in the preparation is therefore a compromise. The product in these preliminary experiments was contaminated with traces of [U-^{14}C]cinnamate. This derived in part from L-[U-^{14}C]phenylalanine originally present; however, it was possible that in both the substrate and the product the 1,4-cyclohexadiene ring was being oxidized to a benzene ring by air. 1,4-Cyclohexadiene (10 mM) added as an antioxidant greatly inhibited the reaction. Near-anaerobic conditions were employed in the preparation, although the necessity for this was not experimentally established. The contaminating cinnamic acid in the product was <4%.

Proof of Structure. The structure of the product is established to be *trans*-3-(1,4-cyclohexadienyl)acrylic acid, **4**, on the basis of its mode of formation, its ^{1}H NMR and UV spectra, its pK_a value, and its identity with the compound obtained by chemical synthesis. The identity has been established by melting point, and by UV, IR, and ^{1}H NMR spectroscopy comparisons (Ressler et al., 1979).

Implications of Steric, Hydrophobic, and Electronic Factors. Various properties of the dihydro acid and L-DiHPhe are relevant to the study of the enzymic reaction. In the Discussion section it will be assumed that the 1,4-cyclohexadiene ring in the dihydro acid and in L-DiHPhe is planar. The NMR spectra of these compounds (this paper and Snow et al., 1968; see also Shoulders et al., 1968) can be accounted for by a planar model or by a dynamic model with enantiomeric boat conformations interchanging at a rate that is fast on the NMR time scale. It has been established, however, after extensive debate, that certain 1,4-cyclohexadiene derivatives do have planar conformations (Rabideau, 1978) and there seems to be no steric reason for postulating that the rings are forced out of planarity in the present instances. The UV spectrum and the physical resemblance of the dihydro acid to cinnamic acid are in keeping with the assumption that the rings are planar. The space-filling properties of the benzene and 1,4-cyclohexadiene rings should be very similar (Figures 3A and 3B).

Table II: Steady-State Catalytic Parameters for the Action of Phenylalanine Ammonia-lyase at pH 8.7 on 3-(1,4-Cyclohexadienyl)-L-alanine Compared with Those Using L-Phenylalanine as a Substrate[a]

source of enzyme	$k_{cat}^{DiHPhe}/k_{cat}^{Phe}$	K_m^{DiHPhe} (mM)	K_m^{Phe} (mM)
maize	0.060	0.16	0.27[b]
potato	0.026	(0.11)[c]	0.26[b]
R. glutinis	0.072	0.55	0.65[b]

[a] Determinations in 0.03 M borate (Na^+) buffer. The limiting kinetic parameters at high substrate concentrations were determined as before (Havir & Hanson, 1968). The reaction was followed spectrophotometrically at 262 nm (ε 15 800). [b] Hanson & Havir, 1977. [c] A low estimate based on inhibition experiments.

The chromatographic evidence suggests that the dihydro acid and cinnamic acid are equally hydrophobic or that the former is slightly more hydrophobic. The same relationship should exist between L-DiHPhe and L-phenylalanine as in partition studies structural differences are found to be additive with respect to the logarithms of partition coefficients (e.g., Leo et al., 1971). The active site region of phenylalanine ammonia-lyase responsible for binding the aromatic ring in substrate and product should be hydrophobic since binding is influenced by the same water entropy effects responsible for locating phenylalanine side chains in proteins (e.g., Manavalan & Ponnuswamy, 1978). Any increase in hydrophobic character in going to the dihydro analogues should lead to stronger binding.

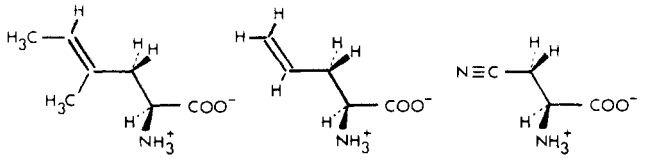
The increase in pK_a of 0.21 pH unit on partially reducing the benzene ring of cinnamic acid (Figure 1) is similar to the effect of an electron-donating para substituent such as $-CH_3$. Introducing a *p*-methyl group into cinnamic acid increased the pK_a by 0.12 pH unit (see legend to Table I), slightly less than the shift of 0.170 unit reported for the para methylation of benzoic acid (Leffler & Grunwald, 1963). With respect to the ionization process, therefore, the double bond is less able to delocalize negative charge than the benzene ring. Although the pK_a values of the β hydrogens of L-phenylalanine and L-DiHPhe are unknown, a similar or larger increase in pK_a may be postulated on going from the former to the latter. The overall equilibrium constant for the elimination reaction L-$Phe^{+-} \rightleftharpoons Cin^- + NH_4^+$ is $^{+-}K = 4.3$ M (see Hanson & Havir, 1972). The pK_a values for carboxyl and amino groups should be the same for phenylalanine and L-DiHPhe; hence on going to the dihydro compounds log ^{+-}K should be lowered by a pK_a change of 0.2 unit and by a lowering in log ^{+0}K for the nonionized acids (produced by a decrease in resonance energy $^{+0}\Delta H°$). The overall equilibrium should thus be moved toward the reactant. The same argument may be applied to the elimination step in the catalytic sequence (see Discussion).

Enzymic Reaction. Table II records steady-state kinetic parameters for the action of phenylalanine ammonia-lyase on L-DiHPhe. In each case the optimum in k_{cat} was at pH 8.7. This is identical with the optimum for L-phenylalanine, but differs from that observed for L-tyrosine (pH 7.7) and for certain other para-substituted L-phenylalanines (Hanson & Havir, 1977). The catalytic process, therefore, is unperturbed with respect to the critical ionizations defining the pH–activity curve (Havir & Hanson, 1968).

The k_{cat} and K_m values listed are estimates of the limiting values at high substrate concentration based on measurements

with 0.5–15 mM substrate. Over this range graphical analysis failed to show any departure from Michaelis–Menten kinetics. The reaction rates were too low to allow measurements at low substrate concentrations in order to determine the extent of negative cooperativity. The k_{cat} values observed were 14–40-fold lower than the corresponding values for L-phenylalanine. The true L-DiHPhe rates may be slightly lower as traces of L-phenylalanine were present in the L-DiHPhe preparation used. The decreases were all appreciably greater than those observed for the para-substituted L-phenylalanines studied previously (**1**, X = OH, F, Cl, Br, I, NO_2); e.g., for the maize enzyme, $k_{cat}^{OH}/k_{cat}^{H} = 0.125$ (Hanson & Havir, 1977). It should be noted that not all substrate modifications result in a reduction of k_{cat}: replacement of the benzene ring by a thiophene ring increased k_{cat} by the factor 1.40 (Hanson and Havir, 1977). The implications of the observed lowering of k_{cat} are considered in the Discussion section.

Table II shows for enzyme from at least two sources that the K_m for L-DiHPhe is slightly less than for L-phenylalanine. Assuming that the catalytic sequence is in quasiequilibrium, then the change probably reflects a change in the dissociation constants for the two substrates. Similarly, the dissociation constant for the dihydro acid appears to be the same or slightly less than for cinnamate. In unpublished experiments (Hanson) binding to the maize enzyme at pH 8.7 has been studied by measuring protection against nitromethane inactivation. Over the 1000-fold ligand concentration range studied, cinnamate and the dihydro acid yielded protection curves that were superposable within the limits of accuracy of the method. In other experiments a comparison was made of the amounts of [U-^{14}C]cinnamate formed from [U-^{14}C]phenylalanine in the presence and absence of cinnamate or the dihydro acid. The latter was inhibitory to the same or a slightly greater extent than the former; e.g., the inhibitions for 8.5 mM dihydro acid and 10 mM cinnamate were: *R. glutinis*, 88:71%; potato, 70:67%; and maize, 52:52%. If the dihydro compounds are somewhat more hydrophobic, as suggested above, slightly stronger binding to the enzyme would be expected (see Discussion).



7 **8** **9**

When compounds **7**, **8**, and **9** were tested as substrates for the enzyme from maize, no increases in UV absorbancy in the 230–290-nm range could be detected (footnote Table III). By making conservative assumptions about the extinction coefficients of the potential ammonia elimination products, one can conclude that the k_{cat} values for these compounds are at least 100-fold lower than the k_{cat} for L-DiHPhe. It appears that, although the compounds contain β-activating groups equivalent to or stronger than the γ,δ-double bond in L-DiHPhe, they are, when bound, misaligned with respect to the catalytic groups on the enzyme. Either the active site is in a productive conformation and the compounds bind in the wrong way, or the active site folds to a nonproductive conformation. Although **7** does not appear to be a substrate for the ammonia-lyase, it will compete for phenylalanine transporting permeases, inhibit the phenylalanine sensitive 3-deoxy-L-arabinoheptusonic acid 7-phosphate synthase, and act as a substrate for phenylalanine tRNA synthetase (Fowden, 1974).

Table III: Inhibition of Phenylalanine Ammonia-lyase from Maize by Substrate and Product Analogues[a]

inhibitor	K_I (mM)
3-cyclohexyl-L-alanine	2.3
(2*S*,*E*)-2-amino-4-methylhex-4-enoic acid[b] (7)	30
DL-allylglycine (8)	280
L-3-cyanoalanine (9)	50
trans,trans-sorbic acid (6)[c]	10

[a] The inhibition constants were determined at low substrate concentrations; see Experimental Section. Compounds 7, 8, and 9 were tested as substrates of the enzyme by treating each compound (20 mM) with maize enzyme (40 mU/mL) at 30 °C and pH 8.7 borate (Na^+) buffer (0.03 M). No significant increases in UV absorbancy were observed at 230, 260, and 290 nM. [b] The contaminants leucine and isoleucine were not inhibitory. [c] The listed K_I agrees with the binding constant for **6** determined in protection experiments using CH_3NO_2 as the inactivator (unpublished experiments). As **6** is a cinnamate analogue, however, it could be a noncompetitive inhibitor of the enzyme.

The K_I values in Table III were determined at low substrate concentrations and, for reasons noted in the Experimental Section, should be held to provide only comparative information. The sample of maize enzyme used showed essentially no negative cooperativity: a single K_m of 0.45 mM was determined. The value 0.5 mM was found in conjunction with the experiments recorded in Table III. The K_I for 3-cyclohexyl-L-alanine is higher than these values. This is in keeping with the view that the greater thickness of the chair-form cyclohexane ring compared with the planar benzene or cyclohexadiene rings leads to poorer binding. Similarly the sequence of increasing K_m and K_I values for L-phenylalanine or L-DiHPhe, **7**, and **8** is in keeping with the postulate that a reduction in space-filling properties as shown in Figure 3 leads to poorer hydrophobic bonding.

Discussion

Olefinic β Activation and Metabolism. The results establish that a double bond can provide sufficient β activation to allow the enzymic elimination of ammonia from an amino acid. The turnover number for the reaction, however, is exceedingly low. The k_{cat} for phenylalanine ammonia-lyase is $\sim 3\ s^{-1}$ per active site (Hanson & Havir, 1972, p 144). With L-DiHPhe as the substrate, this is lowered to $<0.2\ s^{-1}$. For such a reaction to be metabolically significant, the total flux would have to be small or the amount of enzyme large. In phenylalanine ammonia-lyase, the unusual prosthetic group may compensate for the poor β activation afforded by an aromatic system as compared with a carboxylate ion. If one assumes that in the course of evolution this mechanism has approached its maximal possible turnover number and that L-DiHPhe as an analogue only differs electronically from L-phenylalanine, then $0.2\ s^{-1}$ may be close to the best turnover number to be expected for an ammonia-lyase with olefinic β activation. It is therefore not surprising that an enzyme of this type has not been encountered in pathways that involve a significant metabolic flux. The occurrence of such enzymes in pathways for the biosynthesis of secondary natural products in plants is conceivable but not very probable.

Dominance of Electronic Effects. The above argument depends upon the postulate that L-DiHPhe can be treated simply as an electronically modified L-phenylalanine. Changes in the individual rate constants in a catalytic sequence may be separated into steric, hydrophobic, and electronic factors (Hansch et al., 1972). This linear free energy assumption may be extended to allow changes in k_{cat} and K_m to be discussed in terms of the three factors provided the enzymic reaction

is in quasiequilibrium (Hanson & Havir, 1977). As argued previously, the quasiequilibrium assumption is probably justified for phenylalanine ammonia-lyase. It is possible, therefore, to discuss the three types of effect on k_{cat} by reference to the steps most likely to be influenced by the different factors. If these steps are insensitive to changes in steric and hydrophobic properties, the chances are good that the observed changes in k_{cat} are mainly attributable to electronic effects. The argument does not require a detailed knowledge of the kinetics as the conclusion will be the same if the steps believed to be insensitive do not in fact enter into the expression for k_{cat}.

Steric Effects. Any step in the catalytic sequence may be influenced by steric effects, but, if binding leads to misalignment with respect to the catalytic groups, then the rates of the chemical steps will be greatly changed. The open chain compound **7** should be a substrate as it contains a γ–δ double bond but on binding no reaction appeared to take place. The large structural change (Figures 3A and 3C) appears to result in complete misalignment. On the other hand, a linear regression study indicated that k_{cat} was only lowered about 25% when the size of para substituents of L-phenylalanine were increased by 1 Å (Hanson & Havir, 1977). Some misalignment may be produced by the slightly greater bulk of the planar cyclohexadiene ring as compared with the benzene ring but it seems reasonable to postulate that this produces only a small decrease in k_{cat} and does not change the rate-limiting step.

Hydrophobic Effects. These are most likely to influence the substrate binding step and the release of cinnamate or its dihydro analogue. As noted in the Results section, the observed lowering of K_m could be attributed to slightly stronger hydrophobic binding rather than to a steric or electronic effect. The equilibrium constant for binding does not influence k_{cat}; hence hydrophobic effects should not influence k_{cat} unless release of cinnamate or its analogue is rate limiting. (Subsequent steps associated with the hydrolysis of the amino enzyme intermediate are unlikely to be rate limiting: Hanson & Havir, 1977.) As the binding constants for cinnamate and its analogue are essentially the same, it seems unlikely that there is any significant difference in k_{off} for the two compounds or that the rate-limiting step could be changed by hydrophobic factors.

Mechanistic Considerations. If neither steric nor hydrophobic effects are large enough to account for the observed lowering of k_{cat}, then one must ask whether an electronic effect would be expected to lower rather than increase k_{cat}. Although the details of the catalytic sequence are unknown, alternative postulates may be considered. If the electronic effects provide a sufficient explanation for the observed lowering, this supports the above argument concerning mechanistic limitations on enzyme evolution. At the same time, alternative views of the catalytic mechanism are defined for future exploration.

The major point in the sequence likely to be influenced by an electronic effect is C–H bond breaking. In the normal sequence this is probably an equilibrium step prior to the rate-limiting step (low ^3H isotope effect: Wightman et al., 1972). The rate-limiting step could be C–N cleavage if the reaction is stepwise, or subsequent to this. Two possibilities will be considered:

(a) On going to the analogue C–H bond breaking becomes rate limiting. The lowering of k_{cat} observed would then be less than the total lowering of the rate constant for C–H cleavage. As noted in the Results, the acidity of the β hydrogens must be lower in the analogue than in L-phenylalanine. The lowering in k_{cat} is thus explicable as an electronic effect.

(b) There is no change in rate-limiting step on going to the analogue. If the rate-limiting step is subsequent to the elimination step (which could be stepwise or concerted), the equilibrium constant for E(CH—CZ) ⇌ E(HZ)(C═C) enters into the expression for k_{cat} (Z represents the combination of the prosthetic group with the amino group). As noted in the Results, this equilibrium should be shifted by electronic factors toward the left-handed side on going to the analogue. Such a shift would lower k_{cat}. This is in keeping with conclusions reached tentatively in studying the para-substituted phenylalanines: the regression results implied a positive Hammett ρ for the elimination step (Hanson & Havir, 1977).

A more general discussion of conceivable changes in the kinetics of the elimination step is possible with the aid of a free-energy-surface diagram (Hanson & Havir, 1972; More O'Ferrall, 1970). The present results do not allow one to choose between alternative kinetic mechanisms but it appears that the electronic effect provides a sufficient explanation for the observed lowering of k_{cat}.

In the context of the above discussion of the elimination mechanism a negative observation needs to be recorded. If C–N cleavage leads to a carbonium ion intermediate when L-DiHPhe is the substrate, a rearrangement by hydride transfer could occur to generate in the ring a conjugated allylic carbonium ion. The presence at the active site of such a species might lead to enzyme inactivation, but no significant inactivation was observed.

Conclusion

The present study is the first attempt to obtain detailed information at the enzyme level concerning the replacement in a substrate of a benzene ring by a 1,4-cyclohexadiene ring. Hitherto only the pharmacological or antimicrobial consequences of this replacement have been examined. The approach should be of value in the study of other reactions in which an enzyme catalyzes a transformation at a center adjacent to a benzene ring.

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References

Allan, J. L. H., Jones, E. R. H., & Whiting, M. C. (1955) *J. Chem. Soc.*, 1862–1873.

Baden, H. P., Pathak, M. A., & Butler, D. (1966) *Nature (London) 210*, 732–733.

Charm, S. E., & Matteo, C. C. (1971) *Methods Enzymol. 22*, 476–556.

Eisner, U., Elvidge, J. A., & Linstead, R. P. (1953) *J. Chem. Soc.*, 1372–1379.

Elvidge, J. A., & Ralph, P. D. (1966) *J. Chem. Soc. B*, 243–244.

Fowden, L. (1974) *Recent Adv. Phytochem. 8*, 95–122.

Fowden, L., & Smith, A. (1968) *Phytochemistry 7*, 809–819.

Hansch, C., Schaeffer, J., & Kerley, R. (1972) *J. Biol. Chem. 247*, 4703–4710.

Hanson, K. R., & Havir, E. A. (1972) *Enzymes, 3rd Ed. 7*, 75–166.

Hanson, K. R., & Rose, I. A. (1975) *Acc. Chem. Res. 8*, 1–10.

Hanson, K. R., & Havir, E. A. (1977) *Arch. Biochem. Biophys. 180*, 102–113.

Hanson, K. R., & Havir, E. A. (1978) *Recent Adv. Phytochem. 12*, 91–137.

Havir, E. A., & Hanson, K. R. (1968) *Biochemistry 7*, 1904–1914.
Havir, E. A., & Hanson, K. R. (1970) *Methods Enzymol. 17A*, 575–581.
Havir, E. A., & Hanson, K. R. (1973) *Biochemistry 12*, 1583–1591.
Havir, E. A., Reid, P. D., & Marsh, H. V., Jr. (1971) *Plant Physiol. 48*, 130–136.
Hirschmann, H., & Hanson, K. R. (1971) *Eur. J. Biochem. 22*, 301–309.
Honig, B., Warshel, A., & Karplus, J. (1975) *Acc. Chem. Res. 8*, 92–100.
IUPAC-IUB, Recommendations, 1974; Nomenclature of α-Amino Acids (1975) *Biochem. J. 149*, 1–16.
Jackman, L. M. (1959) *Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry*, pp 57–58, 134, Pergamon, New York.
Jones, E. R. H., Mansfield, G. H., & Whiting, M. C. (1956) *J. Chem. Soc.*, 4073–4082.
Kosower, E. M., Closson, W. D., Goering, H. L., & Gross, J. C. (1961) *J. Am. Chem. Soc. 83*, 2013–2015.
Leffler, J. E., & Grunwald, E. (1963) *Rates and Equilibria of Organic Reactions*, p 173, Wiley, New York.
Leo, A., Hansch, C., & Elkins, D. (1971) *Chem. Rev. 71*, 525–616.
Manavalan, P., & Ponnuswamy, P. K. (1978) *Nature (London) 275*, 673–674.
Moore, S. (1968) *J. Biol. Chem. 243*, 6281–6283.
More O'Ferrall, R. A. (1970) *J. Chem. Soc. B*, 274–277.
Nagarajan, G. R., Diamond, L., & Ressler, C. (1973) *J. Org. Chem. 38*, 621–624.
Nari, J., Mouttet, Ch., Fouchier, F., & Ricard, J. (1974) *Eur. J. Biochem. 41*, 499–515.
Nielson, A. T. (1957) *J. Am. Chem. Soc. 22*, 1539–1548.
Rabideau, P. W. (1978) *Acc. Chem. Res. 11*, 141–147.
Ressler, C. (1972) *J. Org. Chem. 37*, 2933–2936.
Ressler, C., Goodman, F. J., Tsutsui, R., & Masakazu, T. (1979) *J. Org. Chem. 44* (in press).
Ricard, J., Nari, J., & Mouttet, Ch. (1972) *Fed. Eur. Biochem. Soc., Meet., 8th 25*, 375–386.
Scott, A. I. (1964) *Interpretation of the Ultraviolet Spectra of Natural Products*, p 443, Pergamon, Oxford.
Shoulders, B. A., Gipson, R. M., Jandacek, R. J., Simonsen, S. H., & Shive, W. (1968) *J. Am. Chem. Soc. 90*, 2992–2993.
Snow, M. L., Lauinger, C., & Ressler, C. (1968) *J. Org. Chem. 33*, 1774–1780.
Whiting, G. C. (1966) *Chem. Ind.*, 1030–1031.
Wightman, R. H., Staunton, J., Battersby, A. R., & Hanson, K. R. (1972) *J. Chem. Soc., Perkin Trans. 1*, 2355–2364.
Williams, D. H., & Fleming, I. (1966) *Spectroscopic Methods in Organic Chemistry*, p 128, McGraw-Hill, New York.
Williamson, K. L., & Johnson, W. S. (1961) *J. Am. Chem. Soc. 83*, 4623–4627.

Heterogeneity of δ-Crystallins of the Embryonic Mallard Lens. Correlation between Subunit Compositions and Isoelectric Points†

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ABSTRACT: δ-Crystallins from the lenses of embryonic mallards (*Anas platyrhynchos*) were analyzed with respect to native and subunit molecular weight, subunit composition, and isoelectric point. $NaDodSO_4$–urea–polyacrylamide gel electrophoresis showed that unfractionated mallard δ-crystallins are composed of approximately equal amounts of subunits with molecular weights near 47 000 and 48 000. Agarose gel chromatography showed that the embryonic mallard δ-crystallins have native molecular weights slightly less than 200 000. Thus, embryonic mallard δ-crystallins appear to be tetramers. Five major and nine minor δ-crystallins were resolved by isoelectric focusing. The five predominant δ-crystallins each cross-reacted with antichick δ-crystallin antiserum, and each had a different proportion of the larger and smaller subunits, indicating a direct relationship between the isoelectric point and the subunit composition. The presence of numerous, minor species of native δ-crystallins with different isoelectric points suggested that the subunits possess charge heterogeneity as well as size heterogeneity.

δ-Crystallin, also called F.I.S.C. (first important soluble crystallin), is one of the major structural proteins of avian and reptilian lenses [see Clayton (1974) for a review]. Most of our knowledge of δ-crystallin comes from investigations on the chicken lens, where it was first discovered (Rabaey, 1962). Embryonic chick lenses are a particularly good source of δ-crystallin, since they are easily obtained and δ-crystallin represents 60–80% of the soluble protein present in the lens (Rabaey, 1962; Genis-Galvez et al., 1968; Yoshida & Katoh, 1971; Piatigorsky et al., 1972).

One important, unexplained finding concerning δ-crystallins of chickens is that the native proteins can be resolved into at least seven species by isoelectric focusing in a polyacrylamide gel (Bours & van Doorenmaalen, 1970; Brahma & van der Starre, 1975) or in free solution (Bours, 1976). The isoelectric points of the components are clustered between a pH of approximately 5.05 and 5.34, with the different forms being separated from each other by only 0.02–0.06 of a pH unit (Bours, 1974, 1976). It is not known if the difference in the isoelectric points of the different δ-crystallins of the chicken lens involves differences in conformation and/or differences in subunit composition. The second possibility is supported

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