L-Phenylalanine Ammonia-lyase. II. Mechanism and Kinetic Properties of the Enzyme from Potato Tubers*

Evelyn A. Havir and Kenneth R. Hanson

ABSTRACT: The properties of the above enzyme (EC 4.3.1.5) have been investigated using the purified species of mol wt *ca.* 330,000 (Havir, E. A., and Hanson, K. R. (1968), *Biochemistry* 7, 1896 (this issue; paper I)). The equilibrium constant for the reaction at 30°, pH 6.8, and zero ionic strength is estimated to be 4.7 *M* ($\Delta F^\circ = -0.93$ kcal/mole). The trend in the ΔF° values for the groups of ammonia-lyase-catalyzed reactions that yield a double bond conjugated with: (1) a thioester (highest ΔF°), (2) two carboxylate ions, and (3) an aromatic group plus a carboxylate ion (lowest ΔF°) may be attributed to resonance. Sulfhydryl reagents failed to inhibit the enzyme and no metal ion requirement was demonstrated. A light-absorbing prosthetic group such as pyridoxal phosphate appeared to be absent. Carbonyl reagents, however, did inhibit the enzyme and the inhibition by $NaBH_4$ was countered by L-phenylalanine and cinnamate. A reactive carbonyl group on the enzyme is considered to take part in the catalytic process: a carbonyl–amine intermediate gives rise to cinnamate and a carbonyl–ammonia intermediate. Hydrolysis of the carbonyl–ammonia group yields free NH_4^+. Kinetic evidence for the formation of an enzyme–ammonia intermediate is presented. The bell-shaped curve for initial velocity at saturating substrate concentrations (V_{satn}) as a function of pH is defined by the constants $pK_\beta = 7.25$ and $pK_\alpha = 10.25$ (optimum pH 8.75). At the pH optimum the activation energy for $V_{satn} = 13.7 \pm 0.5$ kcal/mole. The kinetics of cinnamate formation are not described by the Michaelis–Menten equation. The apparent K_m increases from 0.038 to 0.26 mM and the apparent V_{max} doubles as the concentration of L-phenylalanine increases. D-Phenylalanine, a very poor substrate for the enzyme, acts as a competitive inhibitor; in the presence of this compound Michaelis–Menten kinetics are observed. These kinetic results are attributed to allosteric interactions rather than to the presence of a series of isozymes. The feedback regulation by cinnamate inhibition of the enzyme's *in vivo* activity is discussed.

L-Phenylalanine ammonia-lyase catalyzes the elimination of ammonium ions from L-phenylalanine to give *trans*-cinnamate. The purification from light-exposed slices of potato tubers of a species of the enzyme with a molecular weight of *ca.* 330,000 was described in paper I (Havir and Hanson, 1968). The present paper describes the properties of this purified enzyme. The enzyme is believed to act at a switching point in metabolism diverting phenylalanine from the general pool of amino acids to phenylpropanoid synthesis and it is possible that its unusual kinetic properties are related to its regulatory function. Particular attention is given to the similarities and differences between the enzyme and the other known carbon–nitrogen lyases.

Experimental Section

Initial Rate Measurements. For the definition of enzyme units, the apparatus used, and the standard assay procedure, see paper I (Havir and Hanson, 1968). In all studies of initial velocity, v, as a function of substrate concentration, [S], *ca.* 40 mU of enzyme was used in a final volume of 3 ml; temperature range 29.9–30.1°. The reaction was started by the addition of enzyme. Spectrophotometric observations at 290 mμ, usually 9, were made every 15 or 30 sec and rates were estimated graphically. All samples of the enzyme used (see Havir and Hanson, 1968) were free of the minor species and had specific activities greater than 500 mU/mg. No loss in activity was detectable in stock solutions (10 mg/ml) stored at 4° for several days and samples stored at −20° for 1 week or more had the same activity on thawing. Under the standard assay conditions, v was proportional to the amount of enzyme protein added. No departure from a straight-line relationship was noted for the range of observation 0–80 mμmoles/min. It is assumed that this property applies when lower concentrations of substrate are used.

Graphical and Computer Analysis of Kinetic Data. In this paper the Michaelis–Menten relationship is written in the form $v = \tilde{V}_{max}[S]/([S] + \tilde{K}_m)$, where $\tilde{V}_{max}$ and $\tilde{K}_m$ are observed constants computed on the basis of the experimental results. In discussing competitive inhibition the constants are written without the tild sign as a particular kinetic model is implied. The term V_{satn} is employed for the maximum initial velocity calculated by graphical extrapolation in the case where the experimental data do not conform to the Michaelis–Menten relationship.

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Kinetic data were first plotted on graphs of v against v/[S]. With such plots the points for a particular substrate concentration are limited to a straight line of slope [S] which passes through the origin (Hofstee, 1959; Coleman, 1965). It follows that when a set of replicate observations of v are made at a single value of the controlled variate [S] the estimated confidence limits for the true mean of v are radially distributed along such lines ([S] is assumed to be without error). Observations which show no obvious trend away from a straight-line distribution over a wide range of v may be assumed to obey the Michaelis–Menten relationship within the limits of experimental error (Dowd and Riggs, 1965).

In all cases where the graphical analysis indicated that the Michaelis–Menten relationship was obeyed, maximum likelihood values for the constants, estimates of the standard error about the fitted line, and 50, 75, 90, and 95% confidence limits were computed according to the theoretical treatment of Bliss and James (1966). The output of the FORTRAN program used has been described (Hanson *et al.*, 1967). The assumption made in using the program that the variance in v is approximately independent of its magnitude was checked by making replicate observations (six or eight) at several values of [S]. The standard deviations of these observations were about 1.2 (standard assay set at 96 for 40 mU of enzyme). This value agreed with the weighted mean (1.20) of the standard errors for the distribution of data about the fitted lines (Figures 4 and 6; $N = 163$). In Figure 5 the slightly larger value of 1.3 was adopted in computing the 95% confidence limits.

Ammonia-Free Enzyme. Sephadex G-25 (Pharmacia, New York, N. Y.) columns (0.5 × 7.5 cm) were prepared in disposable Pasteur pipets and equilibrated with the desired buffer. Enzyme (100 or 200 μl) was added, followed by successive 100-μl portions of buffer. The fraction 0.8–1.2 ml contained 95% of the applied enzyme and less than 0.05% of the applied NH_4^+. The same columns were employed to remove borohydride treated enzyme from the products of borohydride action.

[U-^{14}C]Cinnamate. A solution of L-[U-^{14}C]phenylalanine (50 μCi, 0.136 μmole) in 1 N HCl (0.5 ml) (New England Nuclear Corp., Boston, Mass.) was concentrated to dryness, then treated at 30° for 6 hr with phenylalanine ammonia-lyase (300 mU, sp act. 110 mU/mg) in 0.04 M borate (Na^+) buffer (pH 8.5) (4 ml); the conversion was at least 95%. After adding 4 N H_2SO_4 (0.1 ml) cinnamic acid was extracted with ether (four 2-ml portions) and the extract was washed with water (three 2-ml portions). As considerable losses are encountered if an ether solution of cinnamic acid is evaporated the solution was extracted with NaOH (0.5 ml, 100 μmoles) and the cinnamic acid was freed from any residual phenylalanine by paper chromatography.

Chromatographic Separations of Phenylalanine and Cinnamate. Samples (50–100 μl) from the reaction mixtures were added to small test tubes (6 × 50 mm) containing HCl (40 μmoles), L-phenylalanine (1 μmole), and compensatory amounts of $(NH_4)_2SO_4$ if a constant salt content was required (*e.g.*, 20 μmoles/sample). The contents of each tube were applied to a strip of Whatman No. 3MM paper (1 × 18 in.) and the chromatograms were developed (descending): once with 1-butanol–acetic acid–water (4:1:5, v/v, upper phase) until the solvent front had moved about 3 cm (this separated phenylalanine and cinnamic acid from salts and protein) and twice with benzene–acetic acid–water (6:7:3, v/v, upper phase). Phenylalanine was located in a band (3 cm wide) 1 cm from the origin and cinnamate close to the front. The radioactivity in excised portions of the chromatograms was measured with a scintillation counter. The counts per minute determined in this way were 79–81% of the counts per minute of equivalent samples not applied to the chromatogram. In measuring incorporation of labeled cinnamate into phenylalanine (Figures 1, 2, and 8) the cinnamate radioactivity of the samples was calculated by difference. A zero NH_4^+ control was employed to give the true base line. In measuring the rate of cinnamate formation from labeled D-phenylalanine the cinnamate radioactivity was measured.

Gas–Liquid Partition Radiochromatography. Labeled phenylalanine formed from NH_4^+ and tracer amounts of [U-^{14}C]cinnamate were separated from cinnamate by paper chromatography, an aliquot (30,000 cpm) was diluted with carrier phenylalanine (2 mg), and the trimethylsilyl derivative was prepared (Klebe *et al.*, 1966). The products were separated on a 4-ft column (coiled copper, 0.25-in. o.d.) of 5% silicone gum rubber (SE 30) on Anakrom SD 90–100 mesh (Analabs, Hamden, Conn.) with a Perkin-Elmer 810 gas chromatograph (Norwalk, Conn.); column, 170°; injector, 350°; argon carrier gas, 75 cc/min; radioactivity monitor from Barber Colman (Rockford, Ill.). The mass peak and radioactivity peak in the effluent records exactly coincided.

Results

A. Equilibrium Measurements. REVERSIBILITY. The reversibility of the ammonia-lyase reaction was demonstrated with the aid of [U-^{14}C]cinnamate of high specific activity and relatively high concentrations of ammonium ions, *e.g.*, 0.4 M. The difficulty previously experienced in demonstrating this reaction (Koukol and Conn, 1961) must be attributed to an unfavorable combination of rate constants, as under suitable conditions 6% or more conversion may be achieved.

The labeled L-phenylalanine formed was identified by cochromatography on paper, by gas–liquid partition radiochromatography, and by treating the labeled compound with the L-phenylalanine decarboxylase of *Streptococcus faecalis* (Sigma Chem. Co., St. Louis, Mo.) according to the procedure of Udenfriend and Cooper (1953). The labeled 2-phenylethylamine formed was identified by cochromatography on paper. It is possible that some D-phenylalanine was formed by reversal, but the amount formed must be very small as satisfactory equilibria could be established, and as the rate of conversion of D-phenylalanine to cinnamate is extremely slow relative to the rate for its enantiomer (see below).

STANDARD FREE-ENERGY CHANGES. Experiments showing that a true equilibrium for the ammonia-lyase reaction may be reached at pH 6.8, and 8.5 are recorded in Figure 1. In expt A a new equilibrium was reached by

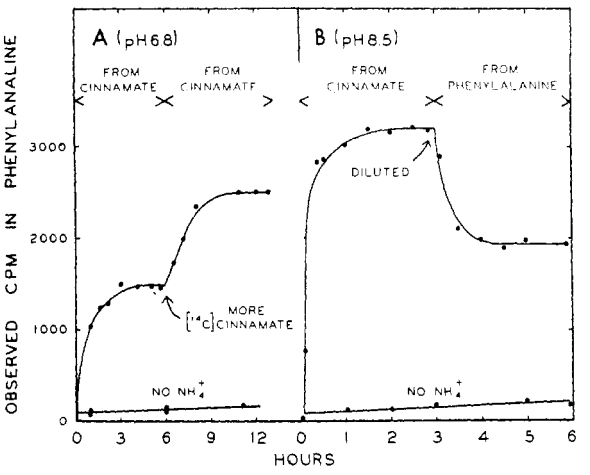


FIGURE 1: Approach to equilibrium. (A) pH 6.8, 30°. The test reaction mixture (800 μl) contained $(NH_4)_2SO_4$ (50 μmoles), phosphate (K^+) buffer (150 μmoles), [U-^{14}C]-cinnamate (586,000 cpm, 1.2 mμmoles), and enzyme (170 mU, sp act. 110 mU/mg). The blank reaction was similar except that the scale was reduced by one-half and $(NH_4)_2SO_4$ was omitted. Ammonia-free enzyme was used in both cases. Aliquots for chromatographic separation (50 μl) and for the determination of total radioactivity were withdrawn at the times shown until 425 μl had been removed. The [U-^{14}C]-cinnamate concentration was then increased by a factor of 1.89 and the NH_4^+ concentration decreased slightly from 0.374 to 0.354 M, by adding 25 μl of the stock solution of labeled cinnamate. (B) pH 8.5, 30°. The test reaction mixture (800 μl) contained $(NH_4)_2SO_4$ (150 μmoles), sodium pyrophosphate–HCl buffer (55 μmoles), [U-^{14}C]cinnamate (1,024,000 cpm, 2.1 mμmoles), and enzyme (170 mU, sp act. 110 mU/mg). The blank reaction was similar; see part A. Aliquots were withdrawn as in part A until 425 μl had been removed. The total ammonia concentration was then reduced from 0.374 to 0.187 M by adding an equal volume of buffer (375 μl). To compensate for this dilution 100 μl rather than 50-μl aliquots were subsequently withdrawn, *i.e.*, the total radioactivity per aliquot was unchanged. The fall in radioactivity shown, therefore, corresponds to a conversion of phenylalanine into cinnamate.

adding more [U-^{14}C]cinnamate, in expt B by diluting the system. The enzyme was still active at the end of these experiments. In other experiments the ability of additional ammonium ions to displace the equilibrium was established. Adding more enzyme was equivalent to adding an equal volume of water.

In order to determine the true thermodynamic equilibrium constant for the elimination reaction it is necessary to consider the effect of pH and ionic strength on the apparent constant, K_{app}, which is defined in terms of the total concentrations (indicated by subscript T) of reactants and products irrespective of their states of ionization or true activities, K_{app} = [ammonium$_T$][cinnamate$_T$]/[phenylalanine$_T$].

In Figure 2, the observed equilibrium ratio of [phenylalanine$_T$]/[cinnamate$_T$] is shown as a function of [ammonium$_T$]. The experiments indicate that K_{app} falls from about 7 or 8 to 5 M as the ammonium$_T$ concentrations decrease. The tangential values represent K_{app} in buffers of 0.4 ionic strength or greater.

The true thermodynamic equilibrium constant for the elimination reaction L-phenylalanine$^{\pm}$ = *trans*-cinnamate$^-$ + NH_4^+ can be estimated from the measure-

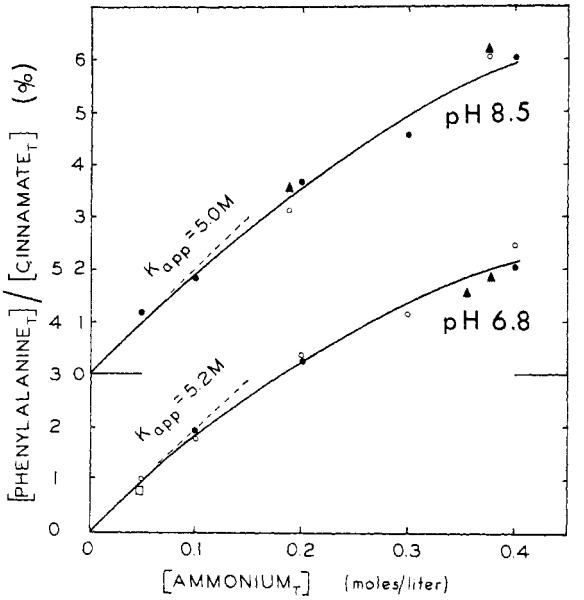


FIGURE 2: Effect of ammonium ion concentration on the observed equilibrium at 30°. The data are derived from a series of experiments including those shown in Figure 1, each experiment at each pH being indicated by a separate symbol. Ammonium ions were added as $(NH_4)_2SO_4$. At pH 6.8 the phosphate (K^+) buffer concentrations were: (●) 0.12 M, (○) 0.08 M, (▲) 0.19 M, and (□) 0.13 M. At pH 8.5 the sodium pyrophosphate–HCl buffer concentrations were: (●) 0.08 M, and (▲ and ○), lower values, 0.084 M and higher values, 0.069 M. The apparent equilibrium constant for elimination, K_{app}, for any point on the curves is given by the reciprocal of the slope of the line passing through the point and the origin. The values of K_{app} indicated are the tangential values at zero [ammonium$_T$].

ments at pH 6.8, as this pH is well removed from the pK_a values for the carboxyl and amino groups, and for ammonium (*e.g.*, see Greenstein and Winitz, 1961). If the available data is extrapolated to low ionic strength, then K is of the order of 4.7 M, *i.e.*, the standard free-energy change $\Delta F^\circ = -RT \ln K \approx -0.93$ kcal/mole (30°).

The finding that K_{app} is essentially the same at pH 8.5 as at 6.8 is not unexpected. If the pK_a for the NH_3^+ group of phenylalanine and that for NH_4^+ are identical, then thermodynamic bookkeeping requires that ΔF° for the reaction L-phenylalanine$^-$ = *trans*-cinnamate$^-$ + NH_3 will be identical with ΔF° at neutrality. The published values for the acid dissociation constants are much the same, and in the system studied (30°, high ionic strength, pH 8.5) they must effectively agree.

B. Requirements for Catalytic Activity. The enzyme used in the following experiments had a specific activity of 250 mU/mg or somewhat higher and the results were checked in several instances using enzyme of the highest specific activity. No evidence for the participation of any cofactors has been found.

EFFECT OF SULFHYDRYL REAGENTS. Enzyme was incubated at 30° with the following reagents (final concentrations given) and all the components of the standard assay mixture except L-phenylalanine. After 15 min, the substrate was added (0.2 ml) and the rate of cinnamate formation was determined. The amount of inhibition

TABLE I: Inhibition by Carbonyl Reagents.[a]

Reagent	Concn (mM)	Rel Act. (%)
None		100
NaCN	3	78
	5	55
Semicarbazide	20	65
hydrochloride	30	33
Phenylhydrazine	2	73
hydrochloride	3	43
$NaBH_4$	4	0

[a] The standard assay was performed in the presence of the compounds indicated at 30° with 25 mU of enzyme/cuvet. The reaction was initiated by adding phenylalanine.

observed was slight in all cases: iodoacetate (5 mM), 8%; *p*-mercuribenzoate (1 mM), 9%; and *N*-ethylmaleimide (3 mM), 13%. The small amount of inhibition, obtained with relatively high concentrations of the reagents may well be the result of secondary effects which change the conformation of the enzyme and result in altered activity (Guidotti and Konigsberg, 1964; Kaufman, 1964). It is possible that the sensitivity of the enzyme from barley (Koukol and Conn, 1961) to sulfhydryl reagents is the result of nonspecific interactions which took place during the prolonged assay period used.

EFFECT OF METALS AND CHELATING AGENTS. None of the following compounds when added to the standard assay mixture to give the indicated final concentrations had any significant effect on the activity of the enzyme: $MgCl_2$ (10 mM), $MnCl_2$ (10 mM), NaF (10 mM), and EDTA (5 mM). Adding salts of the monovalent ions Na^+ and K^+ had no effect on the enzyme except for a slight inhibition at very high concentrations, *e.g.*, 0.5 M. At such levels the ionic strength of the medium undoubtedly effects enzyme activity. There was a 74% inhibition by $HgCl_2$ (1 mM) as measured by the standard assay. This reagent is relatively nonspecific as interactions with the sulfhydryl, imidazole, carboxyl, and amino groups of proteins have been reported (Boyer, 1959).

EFFECT OF CARBONYL REAGENTS. The inhibition of the enzyme by the reagents listed in Table I suggests that a carbonyl group is present at the active site. The inhibition by all of the compounds except borohydride was reversed when the treated enzyme was passed through a Sephadex G-25 column and assayed by the standard procedure.

It is unlikely that a carbonyl group is provided by pyridoxal phosphate as the absorption spectrum of the purified enzyme (0.5 mg/ml), both in acetate (Na^+) buffer at pH 5.5 and borate (Na^+) buffer at pH 8.7, showed only the characteristic protein absorption peak with a maximum at 278 mμ (*A* 0.465). The addition of $NaBH_4$ (1 mg/3 ml) did not alter the spectrum in any way.

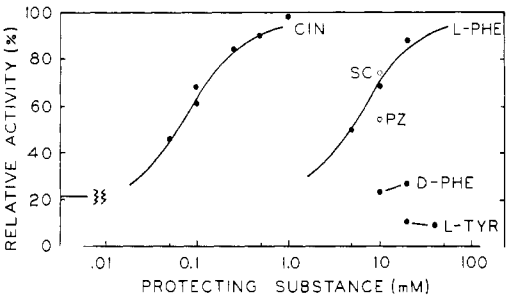


FIGURE 3: Protection against $NaBH_4$ inactivation. CIN, cinnamate; L-Phe, L-phenylalanine; D-Phe, D-phenylalanine; L-Tyr, L-tyrosine; SC, semicarbazide hydrochloride; PZ, phenylhydrazine hydrochloride. Reaction mixture (0.5 ml): $NaBH_4$ (10 μg), phosphate (K^+) buffer (pH 6.8, 50 μmoles), enzyme (53 mU, sp act. 255 mU/mg), and the compound indicated in the figure. In the absence of any protecting compound the enzyme was 79% inactivated. The enzyme was maintained in ice (5 min) with all the components present except borohydride. Borohydride was then added to all the tubes except the no-borohydride control and after a further period at 0° (5 min) the reaction mixture was added to a Sephadex G-25 column in a Pasteur pipet (see Experimental Section). The eluted enzyme (0.6 ml) was assayed under the standard conditions. Experiments were routinely performed at pH 6.8 rather than 8.7 for practical reasons: bubbles did not form in the Sephadex column at the lower pH. In additional experiments with cinnamate (0.10 and 0.25 mM) and L-phenylalanine (20 mM), borohydride was added immediately after these compounds had been added to the enzyme and the reaction mixture was treated as before. The protection observed was unchanged.

In Figure 3 it is shown that L-phenylalanine and cinnamate specifically protected the enzyme at 0°, pH 6.8, from the action of borohydride when the concentration of BH_4^- selected was such that the extent of inactivation was roughly proportional to the amount of borohydride added. Similar protections were observed at pH 8.7. The treated enzyme was separated from the other constituents of the reaction mixtures before being assayed.

The protection afforded by these two compounds appears to be a direct consequence of their ability to bind to the enzyme and not an effect on the hydrolysis of borohydride. There is a rough correlation between the concentrations giving protection and the concentrations of cinnamate (0.1 mM and above) which appreciably inhibit the forward reaction for the enzyme (Figure 4). The protection by cinnamate was not dependent upon the presence of NH_4^+ ions. Concentrations of L-phenylalanine of the same order as those required to saturate enzyme (Figure 5) were needed to protect the enzyme. Protection by L-phenylalanine is not to be attributed to the presence of enzymatically formed cinnamic acid as equivalent results were obtained when borohydride was added at the same time as L-phenylalanine and when addition was delayed for 5 min. L-Tyrosine, which is neither a competitive inhibitor nor a substrate, did not protect the enzyme. Only the results with D-phenylalanine are out of line in that this compound, which is a good competitive inhibitor (Figure 6) and must therefore bind to the enzyme, gave little or no protection. The finding that phenylhydrazine and semicarbazide pro-

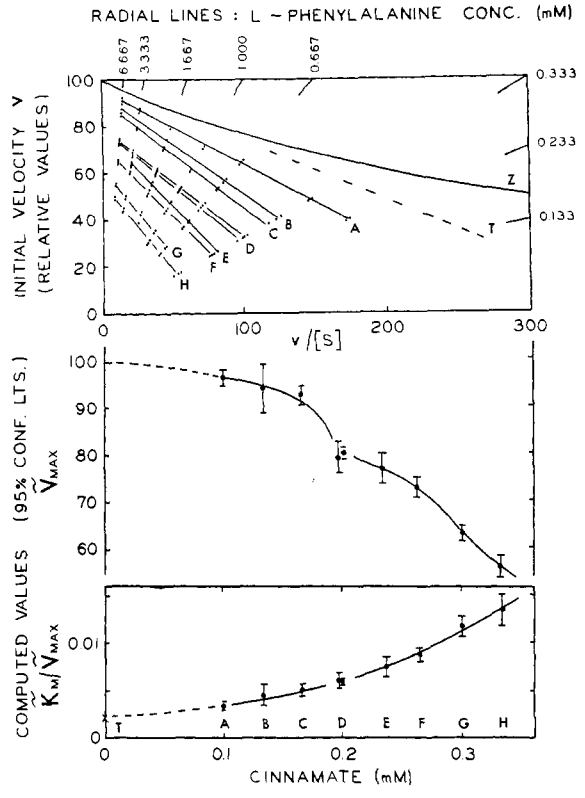


FIGURE 4: Inhibition of cinnamate formation from L-phenylalanine by cinnamate. Temperature 30°, pH 8.7, buffer 0.033 M borate (Na^+). Upper panel: curve Z as in Figure 5 for L-phenylalanine, T is the tangent to this curve at V_{satn}, and curves A–H were determined at the concentrations of cinnamate indicated on the abscissa of the lowest panel. The standard errors in v were computed as 1.6, 2.28, 1.40, 1.45 (0.88), 1.16, 1.30, 0.71, and 1.28 for these separate determinations (N = 18, 6, 11, 10 (23), 8, 18, 10, and 15). The computed values of $\tilde{V}_{max}$ and $\tilde{K}_m/\tilde{V}_{max}$ together with their 95% confidence limits are shown in the middle and lowest panel. The curves in these panels are merely lines drawn through the limits and not theoretical curves.

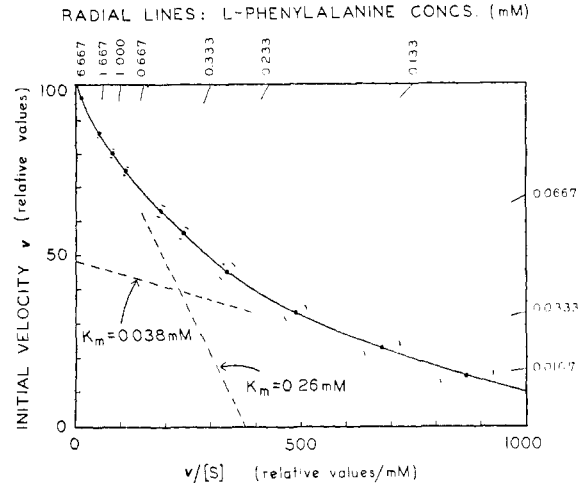


FIGURE 5: Initial velocity of cinnamate formation as a function of L-phenylalanine concentration. Temperature 30°, pH 8.7, buffer 0.033 M borate (Na^+). Values of v are expressed relative to v for the standard assay which is set at 96. This adjustment makes V_{satn} = 100. The points shown are the means of two to six observations. The 95% confidence limits, indicated by radially distributed brackets, are calculated as plus and minus twice the standard deviations of the means on the assumption that the standard deviation of v is constant and equal to 1.3.

tect the enzyme indicates that borohydride and these compounds both react with the same functional group on the enzyme.

C. Kinetic Properties. pH–ACTIVITY RELATIONSHIP. The dependence of the initial velocity of cinnamate formation on pH is shown in Figure 7. The concentration of substrate employed (6.67 mM) was that used in the standard assay (pH 8.7) and v is therefore expressed relative to the standard assay value. The curve for V_{satn} (the saturation value of v at a given pH) must lie slightly above the empirical points (at pH 8.7, 22°, v was 97% of V_{satn} and at pH 6.5, 77% of V_{satn}). The solid curve in Figure 4 is an estimate of the variation of V_{satn} with pH adjusted so that V_{satn} at the optimum pH is 100, and calculated according to eq 1, pK_β = 7.25, pK_α = 10.25, optimum pH 8.75.The curve with a spread between the

$$V_{satn} = 100(1 + 2/K_\alpha/K_\beta)/([H]/K_\beta + 1 + K_\alpha/[H]) \quad (1)$$

pK_β and pK_α values of 3.2 instead of 3.0 pH units appeared to be too broad. Emphasis has been placed on the phosphate and borate buffer points. The glycine buffer points appear to be slightly displaced. The full range of pyrophosphate buffer concentrations has not been investigated, but in the optimum region phosphate, borate, and pyrophosphate buffers give similar values.

The general shape of the pH curve indicates that one or more forward steps in the catalytic reaction sequence require the donation of a proton and one or more steps require the abstraction of a proton; donation and abstraction may or may not be associated with the same "bottleneck" step (Bloomfield and Alberty, 1963).

ACTIVATION ENERGY. An approximate value for the Arrhenius activation energy, μ, at the optimum pH for the composite kinetic term V_{satn} was obtained from nine measurements of v at various temperatures over the range 20–32°. The L-phenylalanine concentration (6.67 mM) was that employed for the standard assay and it was found that the ratio of v, measured under these conditions, to V_{satn} is essentially the same at 23 and 30° (found 97 and 96%, respectively). From a linear regression of ln v against $1/T$, where T is the absolute temperature, μ = 13.7 ± 0.5 kcal/mole. In terms of the absolute reaction rate theory, the apparent heat of activation $\Delta H^{\ddagger} = \mu - RT$ = 13.1 ± 0.5 kcal/mole at 30°.

INITIAL VELOCITY AND SUBSTRATE CONCENTRATION. In Figure 5 the failure of kinetic measurements at the optimum pH to satisfy the Michaelis–Menten relationship is shown by the departure of the experimental points from a straight line. At low substrate concentrations the danger exists that v will be underestimated as a result of product inhibition, but such underestimation would tend to reduce the curvature shown in Figure 5, not enhance it. Essentially the same curve has been obtained with several preparations of enzyme. Qualitatively the results may be described as follows. At substrate con-

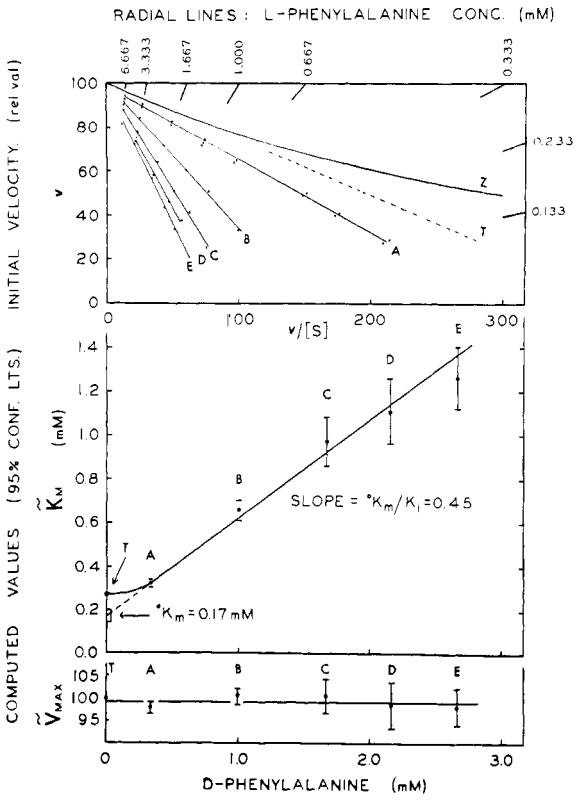


FIGURE 6: Inhibition by D-phenylalanine of cinnamate formation from L-phenylalanine. Temperature 30°, pH 8.7, buffer 0.033 M borate (Na^+). Upper panel: curve Z as in Figure 5 for L-phenylalanine alone. *T* is the tangent (K_m = 0.26 mM) to this curve at V_{satn}. Curves A–E were determined at the concentrations of D-phenylalanine indicated on the abscissa of the lowest panel. The standard errors in *v* were computed as 1.00, 0.68, 1.06, 1.21, and 1.00 for these separate determinations (N = 19, 6, 6, 7, and 6). The computed values for $\tilde{K}_m$ and $\tilde{V}_{max}$ and their 95% confidence limits are shown in the lower panels. The linear regression line in the middle panel is weighted according to 1/(95% confidence limits)^2 for the several $\tilde{K}_m$ values and was computed with the aid of a program written by members of the Yale Computer Center Staff.

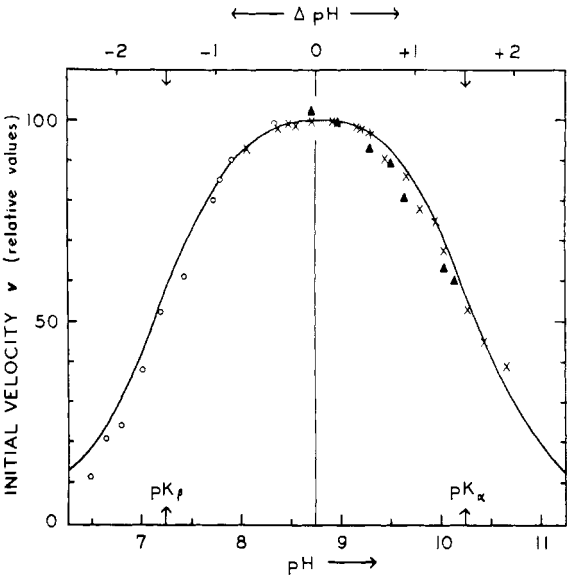


FIGURE 7: Enzyme activity as a function of pH. The experimental points were determined at near saturating concentrations of substrate, *i.e.*, 6.67 mM L-phenylalanine. The temperature was 22° and the buffers 0.033 M: (O) phosphate (K^+), (X) borate (Na^+), and (▲) glycine-NaOH. The pH determinations were made with a Photovolt Model 110 pH meter (Photovolt Corp., New York, N. Y.). The smooth curve represents a theoretical relationship between V_{satn} and pH and is constructed according to eq 1.

centrations of the order of 0.01 mM the enzyme behaves as if it had a K_m of 0.038 mM, but as [S] increases the catalytic efficiency of the enzyme changes until at L-phenylalanine concentrations of the order of 1 mM the apparent K_m = 0.26 mM and V_{max} is twice that anticipated from the data at low substrate concentrations.

D-PHENYLALANINE AS A SUBSTRATE. When 6.67 mM D-phenylalanine was treated with 40 mU of enzyme at 30° under the standard assay conditions no detectable change in ultraviolet absorption took place in 15 min. The rate for D-phenylalanine is thus at least $^1/_{200}$th that for L-phenylalanine. When 0.5 mM D-[1-^{14}C]phenylalanine (New England Nuclear, Boston, Mass.) was treated with 150 mU of enzyme at 30° and pH 8.7 and samples were withdrawn at hourly intervals for 5 hr, a steady rate of [1-^{14}C]cinnamic acid formation was observed. After 5 hr 3.3% of the substrate had been converted into labeled cinnamic acid. In a similar experiment continued for 23 hr a 20% conversion was observed and the rate of elimination at the end of the experiment was only slightly less than the initial rate. The suppliers of the labeled compound state that the method of preparation and an assay for the L-contaminant using L-amino acid oxidase establish that the content of this enantiomer is less than 0.4%. A 20% conversion is thus far too high to be attributed to a contaminant and it would seem to be more reasonable to suppose that D-phenylalanine is an exceedingly poor substrate for the enzyme than that a specific racemase is present.

For experiments with 0.5, 0.33, 0.1, and 0.01 mM D-[1-^{14}C]phenylalanine the observed initial rate of [1-^{14}C]-cinnamate formation compared with the rate for cinnamate formation from L-phenylalanine (Figure 5) decreased as the substrate concentration decreased. At the highest concentration it was $^1/_{5000}$th and at the lowest $^1/_{16,000}$th of the expected rate. The results departed from the Michaelis–Menten equation in the same manner observed for L-phenylalanine. The tangential K_m at high substrate concentration is greater than 0.3 mM, but probably less than 1.0 mM. As the radioactive substrate used undoubtedly contains some L-[1-^{14}C]phenylalanine these experiments probably overestimate the effectiveness of D-phenylalanine as a substrate.

D-PHENYLALANINE AS A COMPETITIVE INHIBITOR. As D-phenylalanine is such a poor substrate it is possible to use the compound as an inhibitor and study the rate of cinnamate formation from L-phenylalanine at various concentrations of its enantiomer. The results of such experiments are shown in Figure 6. The five curves A–E cover D-phenylalanine concentrations of 0.333–2.67 mM. In each case the Michaelis–Menten relationship appears to be obeyed. (The significance of this result is examined in the Discussion.) At least to a first approximation, the

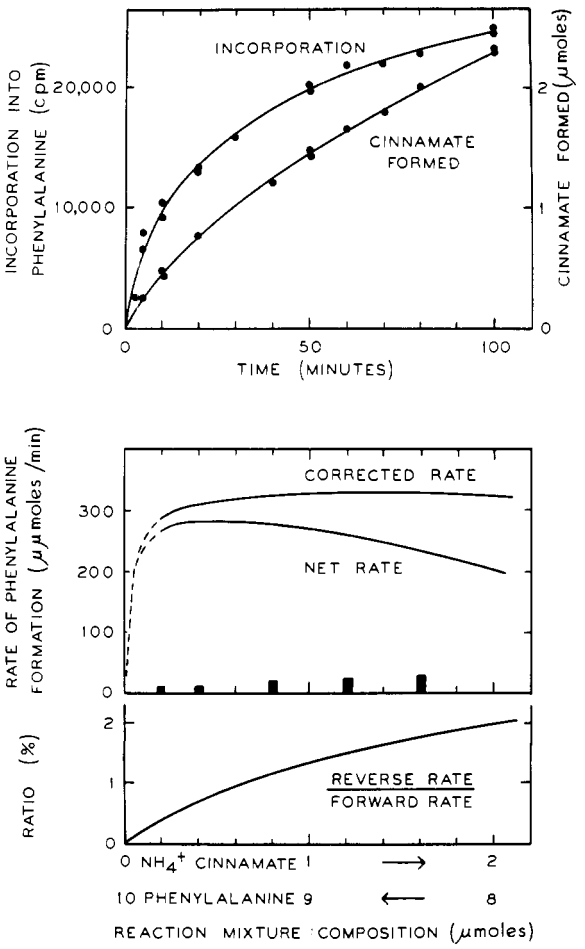


FIGURE 8: Evidence for a partial reaction between an enzyme–ammonia intermediate and cinnamate. The reaction mixture (1 ml) contained L-phenylalanine (10 μmoles), [U-^{14}C]-cinnamate (800,000 cpm, 1.6 μmoles), phosphate (K^+) buffer (pH 6.8, 75 μmoles), and enzyme (300 mU); temperature 30°. Incorporation into phenylalanine: aliquots (25 μl) for chromatographic separation were withdrawn at the times indicated. Cinnamate formed: aliquots (50 μl) were withdrawn, each sample was added to a cuvet containing water (2.45 ml), and the absorbancy at 280 mμ was followed for 3 min. The correction for drift applied to give the absorbancy at the time of sampling was very small. The curves in the lower two panels were calculated as a series of independent points from the curves in the top panel by taking slopes at 10-min intervals. Control: the vertical bars in the middle panel indicate upper bounds to the rates, expressed on a 1-ml basis, for the reverse reaction. The concentrations of buffer and enzyme in the reaction mixtures (0.3 ml) were equivalent to those in the test experiment; the ammonium cinnamate concentrations were as indicated. Each mixture contained [U-^{14}C]cinnamate (170,000 cpm). Aliquots (50 μl) for chromatographic separations were withdrawn at 5, 35, 65, and 95 min.

data conform to the steady-state model for simple competitive inhibition. In the lowest panel it is shown that a straight line may be drawn perpendicular to the ordinate through the 95% confidence limits for the computed values of $\tilde{V}_{max}$, *i.e.*, $\tilde{V}_{max}$ is constant. The middle panel shows that a suitably weighted regression line falls within the 95% confidence limits for the various computed values of $\tilde{K}_m$, *i.e.*, $\tilde{K}_m = {}^{\circ}K_m(1 + [I]/K_i)$, where [I] is the inhibitor concentration, ${}^{\circ}K_m$ is the theoretical Michaelis constant for the enzyme in the absence of inhibitor, and K_i is the dissociation constant for the enzyme–inhibitor complex. From the slope and intercept of the line K_i = 0.38 mM. This value is of the same order as the rough estimate obtained from the experiments with D-[1-^{14}C]-phenylalanine. It would seem, therefore, that D-phenylalanine can bind to the active site, but when the compound is bound the groups on the enzyme are poorly situated to play an effective chemical role.

EVIDENCE FOR AN ENZYME–AMMONIA INTERMEDIATE. If cinnamate can be released from the enzyme before ammonia, then a partial reaction of cinnamate and amino–enzyme can take place to give phenylalanine. In Figure 8 it is shown that when the forward reaction for the enzyme occurs in the presence of labeled cinnamate the formation of phenylalanine from cinnamate takes place at a rate which is much greater than can be explained as an over-all reverse reaction.

The time courses of cinnamate formation from L-phenylalanine (10 μmoles, initial rate *ca.* 85% V_{satn}) and of the concomitant incorporation of radioactivity into L-phenylalanine are shown in the upper panel. The pH of 6.8 was adopted by analogy with the case of histidine ammonia-lyase (Peterkofsky, 1962). The fact that labeled L-phenylalanine was indeed formed was established as in the equilibrium experiments. After 3 min, the cinnamate originally present in the reaction mixture was less than 1% of the total. For the rest of the experiment, therefore, the mixture contained essentially equimolar amounts of ammonium and cinnamate ions. The rates of incorporation of radioactivity into phenylalanine were calculated by taking tangents to the curve for the time course of incorporation. From this information and from the specific activities of cinnamate at these times, net rates of phenylalanine formation were calculated (middle panel). The net rates are lower than the true rates as the rate at which cinnamate is formed from phenylalanine is disregarded.

Upper bounds to the rates for the reverse over-all reaction were obtained at various concentrations of ammonium cinnamate with the aid of labeled cinnamic acid by following the reaction for 90 min (middle panel, vertical bars). The radioactivity associated with phenylalanine in the paper-strip assay did not differ significantly from that of blank determinations, and the increase in height of the bars with increasing ammonium cinnamate concentrations merely reflects the decrease in the specific activity of the cinnamate employed. These calculated maximum values all lie well below the curve for the net rate of phenylalanine formation and large errors in the calculation of this curve could be tolerated without altering the conclusion that the partial reaction rates exceed the reversal rates.

The above argument is open to the objection that the control experiment was not performed under the same conditions as the test. In the test, however, L-phenylalanine would be expected to compete with ammonium and cinnamate for the active site so that the reversal rate would be less than in the control. To eliminate the possibility that phenylalanine induces conformational changes which accelerate the reverse rate it will be necessary to treat L-phenylalanine with enzyme in the pres-

TABLE II: $\Delta F°$ Values for Ammonia-lyase-Catalyzed Reactions.

Amino Acid	Temp (°C)	pH	Mg^{2+}	K^a (M)	$\Delta F°$ (kcal/mole)
β-L-Alanylpantethine[b]	25	7.5	−	1.0×10^{-6}	8.16
L-Aspartate[c]	29	7.2	−	0.0124	2.63
		7.2	−	0.0074	2.76
d	25				3.70
threo-3-Methyl-L-aspartate[e]	25	7.9	+	0.18	1.01
L-Histidine[f]	25	8.0	+	3.0	−0.65
L-Phenylalanine[g]	30	6.8	−	4.7	−0.93

[a] The listed values of K are estimates of the true thermodynamic equilibrium constants for the elimination reactions (products per reactants). Carboxyl groups are assumed to be fully ionized and amino groups and ammonia fully protonated. Calculations based on published data for Mg^{2+} binding by amino acids (*e.g.*, see Greenstein and Winitz, 1961b) establish that the equilibria should not be significantly effected by Mg^{2+} ions under the conditions used in cases *e* and *f*. In the case of histidine ammonia-lyase no correction has been made for the differences in the pK_a values of the imidazole groups of histidine and urocanic acid, but at the pH employed such a correction must be small. The standard free energy of elimination was calculated as $\Delta F° = -RT \ln K$ except in the case *d* which is derived from free energy of formation data. The use of two decimal places follows custom (Burton and Krebs, 1953) and does not indicate accuracy. [b] Vagelos *et al.* (1959); rough value. [c] Results obtained with fumarate hydratase free enzyme by Wilkinson and Williams (1961), $\Delta H° = ca.$ 15 kcal/mole, $\Delta S° = ca.$ 38 kcal/mole deg. See also Williams and McIntyre (1955) and Borsook and Huffman (1938). [d] Burton and Krebs (1953). [e] After Barker *et al.* (1959). The mean value of K for the published equilibrium determinations is 0.238 M. The value listed was obtained by extrapolating the data to low NH_4^+ concentrations. [f] Williams and Hiroms (1967), a preliminary estimate. [g] This paper.

ence of both ^{14}C-labeled cinnamate and ^{15}N-labeled ammonia (*cf.* Peterkofsky, 1962).

The true rates of phenylalanine and cinnamate formation may be calculated iteratively from the experimental results. A first estimate of the true rates of phenylalanine formation is shown in the middle panel (corrected rate). These rates proved to be less than 2% of the net forward reaction (lowest panel).

PRODUCT INHIBITION. Studies of the effect of NH_4^+ ions on the forward reaction indicate that the reaction sequence for the enzyme is not of the Ordered Uni Bi type in which NH_4^+ is released before cinnamate (see Wong and Hanes, 1962; Cleland, 1963). Ammonium ions at concentrations sufficient to provide effective reversal rates had very little influence on the rate of cinnamate formation. The observed changes may be attributed to the change in ionic strength, *e.g.*, when the standard assay was performed in the presence of 0.1 M NH_4Cl a 10% inhibition was observed, but a similar inhibition was observed with NaCl. If NH_4^+ were released before cinnamate then in the theoretical model V_{max} would be expected to decrease asymptotically to $V_{max} = 0$ as $[NH_4^+]$ increased. By analogy V_{satn} should do the same. The partial-reaction experiment described above also eliminates this kinetic model.

The two further possibilities that the sequence is either Ordered Uni Bi (cinnamate before NH_4^+) or Random Uni Bi cannot be distinguished on the present evidence. The results of cinnamate inhibition studies (Figure 4) are exceedingly complex. In the presence of the inhibitor the relationship between v and [S] appeared to conform to that of the Michaelis–Menten equation, but the curves for $\tilde{V}_{max}$ and $\tilde{K}_m/\tilde{V}_{man}$ as a function of cinnamate concentration do not approximate to the curves expected for either of the theoretical models.

Discussion

Equilibrium Constant. The known equilibrium constants and $\Delta F°$ elimination values for ammonia-lyase-catalyzed reaction are listed in Table II. These reactions may be divided on the basis of the types of double bond formed into three groups: (1) conjugation to a thioester group (highest $\Delta F°$), (2) conjugation to two carboxylate ions (middle two values), and (3) conjugation to an aromatic system and a carboxylate ion (lowest two values). In the absence of specific information it is reasonable to consider that $\Delta S°$ has much the same (positive) value for all of these reactions and that the $\Delta F°$ order of these three groups is also the $\Delta H°$ order, *i.e.*, the amount of heat absorbed in transforming, at unit activities, 1 mole of reactant to products is greatest for group 1 and least for group 3. As the three groups differ in the amount of charge separation taking place on elimination the trend in $\Delta H°$ must be influenced by coulombic interactions. If such interactions were the only factor in ranking the groups, group 1 would have the smallest $\Delta F°$ rather than the largest. Also the contribution to the difference between groups 2 and 3 should be small. (The difference in the pK_a values for the α-amino groups of amino acids with one and two carboxyl groups suggests that the coulombic factor is

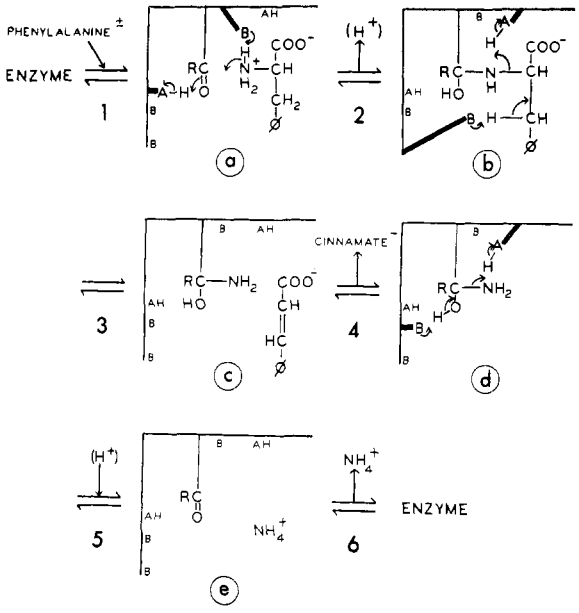


FIGURE 9: Postulated mechanism involving a reactive carbonyl group. The states of ionization shown are assumed to apply at the optimum pH (8.7) for the enzyme. Electron-flow arrows apply to the forward reaction. Ionization processes are assumed to be fast compared with other processes and therefore the loss and gain of H^+ by basic (B) and acidic (A) groups after bifunctional catalysis (steps 2, 3, and 5) and the loss and gain of H^+ in the reaction sequence are not shown as separate steps. The number of B and A groups shown is the maximum number required by the mechanism.

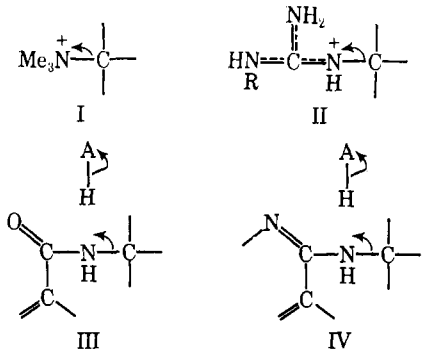
less than 1.4 kcal/mole; see Greenstein and Winitz, 1961). The trend, therefore, may be attributed to the effect of resonance: conjugation with the aromatic system provides the greatest resonance stabilization (lowest heat content) in the series of formed double bonds.

The above analysis suggests that the tyrosine and *m*-tyrosine ammonia-lyase reactions (Neish, 1961) will be found to have ΔF° values as low or lower than that for phenylalanine ammonia-lyase.

As the factors influencing ΔF° for the several ammonia-lyase reactions also influence the rate constants for elimination it is more probable that the enzymes within a group will have similar mechanisms than enzymes from different groups. In the next section the apparent similarity in mechanism between histidine and phenylalanine ammonia-lyase is considered.

Mechanism. The experiments with borohydride and various carbonyl reagents (Table I and Figure 3) suggest that a carbonyl group is involved in the catalytic process. In Figure 9 a possible mechanism for the enzyme's action is shown in which the breakage of the N–C linkage is facilitated through the formation of a carbonyl–amine intermediate (b) which leads to a carbonyl–ammonia intermediate (c). This intermediate is then hydrolyzed. Except in the matter of protonation, the mechanism is analogous to that put forward by Smith *et al.* (1967) to accounts for their studies of histidine ammonia-lyase (see also Peterkofsky, 1962). At the optimum pH of 8.7 the carbonyl–amine would be expected to be largely unprotonated if the analogy

CHART I



of the formol titration is applicable (Kallen and Jencks 1966).

An important aspect of the mechanism may be emphasized by comparing the structure of the carbonyl–amine intermediate with the structures of the substrates for certain other carbon–nitrogen lyases. The partial formulas shown in Chart I correspond to I, ergothionine (Wolff, 1962); II, argininosuccinate (Havir *et al.*, 1965); and III and IV, two compounds which are substrates for the same enzyme (Miller *et al.*, 1959), AICAR (phosphoribosyl-4-(*N*-succinocarboxamide)-5-aminoimidazole), and adenylosuccinate. The elimination of Me_3NH^+ from I appears to be a typical base-catalyzed Hofmann elimination and case II is closely similar. In both N–C bond cleavage is facilitated by the electron deficiency on the nitrogen atom. In cases III and IV electrons are likewise withdrawn from the nitrogen atom so that there is a marked tendency for cleavage and proton acceptance is likely to accompany rather than precede cleavage. For the postulated carbonyl–amine intermediate of Figure 9b, the C–OH group likewise acts to withdraw electrons, but as the nitrogen atom accepts protons more readily than in cases III and IV the actual transition state is probably closer to the situation in which the nitrogen is protonated. To summarize, in cases I–IV electron withdrawal is built into the substrate, whereas in phenylalanine and histidine ammonia-lyases it is built into the enzyme. One may conjecture that metal ions only play a critical role in catalysis for the N–C lyases when nitrogen substitution is not involved (Bright, 1964, 1967; Williams and Lartigue, 1967). For recent general discussions of E2 and E1cB elimination reactions, see McLennon (1967), Bunnett (1962), and references therein.

The proposed mechanism can account for the inactivation of the enzyme by borohydride, its reversible inhibition by certain carbonyl reagents, and the reaction between an amino–enzyme intermediate and cinnamate to form phenylalanine. It will be necessary to show that inactivation is not the result of disulfide bond cleavage (*e.g.*, see Light and Sinha, 1967), to identify the carbonyl group involved in catalysis, and to try and distinguish between the mechanism as written and certain possible variants. For example, the carbonyl–amide could form

a Schiff's base prior to the elimination step. If in the presence of substrate conformation changes limit access to the active site, then a Schiff's base formed between phenylalanine and the enzyme might not be attacked by borohydride (*e.g.*, Horecker *et al.*, 1961). The finding that D-phenylalanine does not protect the active site suggests that the protection afforded by its enantiomer may be chemical and not physical, however cinnamate must physically mask the reducible group from the action of borohydride.

Our experiments on phenylalanine ammonia-lyase and those of Smith *et al.* (1967) on histidine ammonia-lyase indicate that the reactive carbonyl group in these enzymes is not that of pyridoxal phosphate. There are a variety of ways, however, in which a carbonyl group could be derived from a normal amino acid side chain, from an N-terminal amino acid, or by the addition of a small molecule such as glyoxylate to the enzyme. The carbonyl group of pyruvate has recently been shown to catalyze N–C bond cleavage in D-proline reductase (Hodgins and Abeles, 1967). If both histidine and phenylalanine ammonia-lyase involve the same type of carbonyl group, then the possibility must be considered that both enzymes derive from a common, but less specific, ancestral enzyme.

Allosteric Interactions. The departure of the relationship between v and [S] from the Michaelis–Menten equation (Figure 5) may be explained by assuming: (a) that two, or more, different types of active site are present, or (b) that some type of cooperative (allosteric) interaction takes place between constitutionally and sterically equivalent subunits of the enzyme.

If diverse sites are present these could arise because constitutionally distinct subunits are combined in a series of isozymes or because constitutionally equivalent subunits adopt different conformations within the same molecule. They could also arise because the active sites of the native enzyme are modified in a random fashion during the extraction and purification process (*e.g.*, tanning by phenols and phenolase). It is difficult to reconcile the diverse site hypothesis with the finding that the kinetic behavior of the enzyme in the presence of moderate concentrations of D-phenylalanine is described by the Michaelis–Menten relationship (Figure 6). If D-phenylalanine inhibited one of two types of active site very strongly so that only one type was available and subject to competitive inhibition, then simplified kinetics would be observed, but $\tilde{V}_{max}$ for the enzyme in the presence of D-phenylalanine would be significantly less than V_{satn} for the enzyme in its absence. The lowest panel in Figure 6 establishes that this is not the case.

On the other hand a qualitative explanation for the enzyme's behavior can be given on the assumption that allosteric interactions are involved. The following interpretation is based on the model proposed by Monod *et al.* (1965). It remains to be established that this particular model can account for curves of the type shown in Figure 5 as well as the more familiar S-shaped curve. Let it be assumed (a) that all active sites in any given molecule of the enzyme are constitutionally and sterically equivalent and exhibit kinetic properties described by the Michaelis–Menten relationship, (b) that the enzyme may adopt two or more conformations each conformation having its own characteristic catalytic properties, (c) that the equilibrium between the several conformations is a function of the number of sites which under the conditions of the experiment bind L- or D-phenylalanine, and (d) that at high enough concentrations of either of these compounds, a single conformation of the enzyme becomes dominant. It follows that studies of the action of the enzyme on L-phenylalanine in the presence of high concentrations of the D enantiomer will reveal the properties of the enzyme in this single conformation.

The immediate objection to this view is that the intercept, $^{\circ}K_m$ (0.17 mM), for the regression line for $\tilde{K}_m$ against [I] of Figure 6 (middle panel) does not coincide with the tangential value of 0.26 mM calculated for the $\tilde{K}_m$ for L-phenylalanine at high substrate concentrations (Figure 5). Clearly the tangential value, T, is subject to error, and this error cannot easily be estimated, however it is also possible that the sample distribution in determining line A of Figure 6 has failed to reveal that the population being sampled is not defined by the Michaelis–Menten relationship. As the concentration of D-phenylalanine decreases a departure from the relationship must occur. If the true tangential value for $\tilde{K}_m$ at this lowest concentration of D-phenylalanine is higher than the computed value of 0.06 mM, then an excellent straight line may be drawn through T and all the points in the middle panel. In this case $^{\circ}K_m/K_i$ is 0.39, K_i = 0.67 mM, and K_m = 0.26 mM. It should be noted that the disputed point corresponds to a concentration of D-phenylalanine which is less than the estimated K_i.

The above argument does not account for the complexity of the results of cinnamate inhibition (Figure 4). (It is possible that modifier sites which bind cinnamate are present and that this complicates the kinetics.)

Feedback Regulation. The experiments on cinnamate inhibition shown in Figure 4 were carried out at the optimum pH, whereas the physiological pH may be closer to neutrality. In the region of pH 7 cinnamate is much more effective as an inhibitor of its own synthesis. Thus for 1 mM L-phenylalanine at pH 6.6 and 30°, 0.167 mM cinnamate inhibited the enzyme 80% whereas at the optimum pH the inhibition was only 22%. Although the optimum conditions for *in vitro* feedback control have not been established it is reasonable to postulate that the pH and substrate concentrations are such that control takes place, and the system operates to maintain a small but fairly constant pool of cinnamate available for further metabolism. Feedback control would minimize the effect of short-term variations in phenylalanine concentration on cinnamate metabolism. As cinnamate may be channeled to several end products, significant feedback control may be exercised at later branch points in its metabolism.

The possibility for a second type of control is suggested by the present study. If the mechanism shown in Figure 9 applies, then the enzyme's activity could be reversibly altered through the action of a specific dehydrogenase or transaminase acting on the carbonyl

group of the active site. Such control would link phenylpropanoid synthesis to other metabolic changes within the cell.

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