# Deuterium Isotope Effect in Enzymatic Transamination of L-Deuterio-phenylalanine to Deuterio-phenylpyruvic Acid

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**Abstract** □ L-Deuterio-phenylalanine was isolated by gradient elution ion-exchange chromatography of the ionic fraction obtained from the cell wall hydrolysate of algae grown in culture medium containing 99.6% deuterium oxide. The enzyme system present in rat brain extract was used for determining the rate of transamination of L-deuterio-phenylalanine and its protio analog. The phenylpyruvate formed was determined spectrophotometrically at 300 nm. using the arsenate-catalyzed enol–borate assay procedure. Transamination rates were determined in media containing varying concentrations of amino acid and α-ketoglutarate. Kinetic determination of maximum velocity was conducted through graphical analysis of the data. The deuterium isotope effect, expressed as the $V_H/V_D$ value, was 1.64.

**Keyphrases** □ L-Deuterio- and L-protio-phenylalanine—isolation, identification, enzyme transamination to phenylpyruvates, deuterium isotope effect □ Deuterio-phenylpyruvic acid—enzymatic transamination product from L-deuterio-phenylalanine, deuterium isotope effect □ Deuterium isotope effect—L-deuterio-phenylalanine enzyme transamination to deuterio-phenylpyruvic acid □ Transamination rates—L-deuterio- and L-protio-phenylalanine, deuterium isotope effect □ Ion-exchange chromatography—isolation, L-deuterio-phenylalanine □ UV spectrophotometry—monitoring, L-deuterio-phenylalanine transamination

The large-scale cultivation of algae grown in a culture medium containing 99.6% deuterium oxide has made available a source of fully deuterated chlorophyll, carbohydrates, and proteins (1). Hydrolysis of the cell walls of *Scenedesmus obliquus*, followed by ion-exchange chromatography, was reported by Blake *et al.* (2) to yield L-amino acids that are fully deuterated in nonexchangeable positions. L-Deuterio-phenylalanine was shown to be present in the algae hydrolysate in sufficient amounts to permit isolation and purification.

In living systems the transamination reaction is an important biological process involving the transfer of an amino group between certain amino and keto acids. The mechanism of enzymatic and nonenzymatic transamination was reviewed by Snell and Jenkins (3). Deuterium isotope effects in the transamination reaction of L-alanine and L-deuterio-alanine with pyridoxal were studied by Blake *et al.* (4). The effect of the nature and concentration of added buffer, the pH, and the effect of metal ion were included in the study. Lin *et al.* (5) reported the deuterium isotope effect in the nonenzymatic transamination of L-deuterio-glutamic acid. Fang *et al.* (6) performed a kinetic study on enzymatic transamination using four analogs of L-glutamic acid (fully deuterated glutamic acid, two partially deuterated forms of glutamic acid, and protio-glutamic acid) as substrates. The deuterium isotope effect reflected in the transamination rate was determined by maximum velocity analysis.

In the present study, L-deuterio-phenylalanine was isolated from the ionic fraction of the algae cell wall hydrolysate by gradient elution ion-exchange chromatography. The compound was recrystallized from acetone–water and was characterized by TLC; NMR, IR, and mass spectrometry; polarimetric analysis; and elemental analysis. Transamination of the protio and deuterio analogs of L-phenylalanine to the corresponding analogs of phenylpyruvic acid was performed with rat brain extract as the enzyme system. The rate of the reaction was followed spectrophotometrically at 300 nm., using the arsenate-catalyzed enol–borate assay procedure for phenylpyruvate described by George *et al.* (7). The deuterium isotope effect was determined from a comparison of maximum velocity analysis based on the binary mechanism of enzymatic transamination involving L-protio-phenylalanine and L-deuterio-phenylalanine.

## EXPERIMENTAL

**Isolation and Purification of L-Deuterio-phenylalanine**—The ionic fraction obtained from the hydrolysate of the residual cell wall of *Scenedesmus obliquus*, grown autotrophically in a nutrient medium containing 99.6% deuterium oxide (8), was chromatographed in a two-step procedure utilizing the following ion-exchange resins: (*a*) Dowex 50-X8 for concentrating and desalting, and (*b*) Dowex 1-X8 for concentrating and isolating the deuterated dicarboxylic acids, L-deuterio-glutamic and L-deuterio-aspartic acids. A detailed description of this two-step chromatographic procedure was reported earlier (9). Effluent fractions from the second step that were shown to contain L-deuterio-phenylalanine by TLC were combined and evaporated *in vacuo* at room temperature until the residue was free of acetic acid. Since other deuterated amino acids were present in each of the fractions pooled, the isolation of L-deuterio-phenylalanine from this mixture was effected by means of gradient elution chromatography on a column of polystyrene sulfonic acid resin. For this step, Rexyn 101 (453 g.), in the sodium salt form, was hydrated, freed of fines, and packed into a chromatographic column (2.7 × 869 cm.). The resin was subsequently converted to its acid form by washing with 1 *N* HCl. Excess acid was then removed by washing the resin with distilled water until the effluent was neutral.

The performance and efficiency of the separation procedure were determined by conducting preliminary runs using samples containing L-protio-phenylalanine, L-protio-leucine, and a mixture containing equal amounts of L-protio-phenylalanine and L-protio-leucine. The gradient of hydrochloric acid needed to effect complete separation, as well as the elution volumes of the respective amino acids, were determined. The column was then washed with distilled water until the effluent was neutral.

The residue containing the deuterated amino acids was dissolved in 180 ml. of distilled water and then loaded on the column. Elution of the amino acids was started with 1 *N* HCl. The effluent was collected in 27-ml. fractions on an automatic fraction collector[1] at a rate of 55 ml./hr. After elution with 300 ml. of 1 *N* HCl, the hydrochloric acid concentration was increased gradually by feeding 4 *N* HCl dropwise into a 1000-ml. mixing chamber containing the remaining 1 *N* HCl (600 ml.). Elution with this gradient was continued until 5000 ml. of total effluent was collected. Fractions whose elution volumes corresponded with those determined previously for L-protio-phenylalanine and L-protio-leucine were evaporated to dryness at room temperature with the aid of an electric fan, and a small portion of each was subsequently tested with ninhydrin reagent.

[1] Rinco.

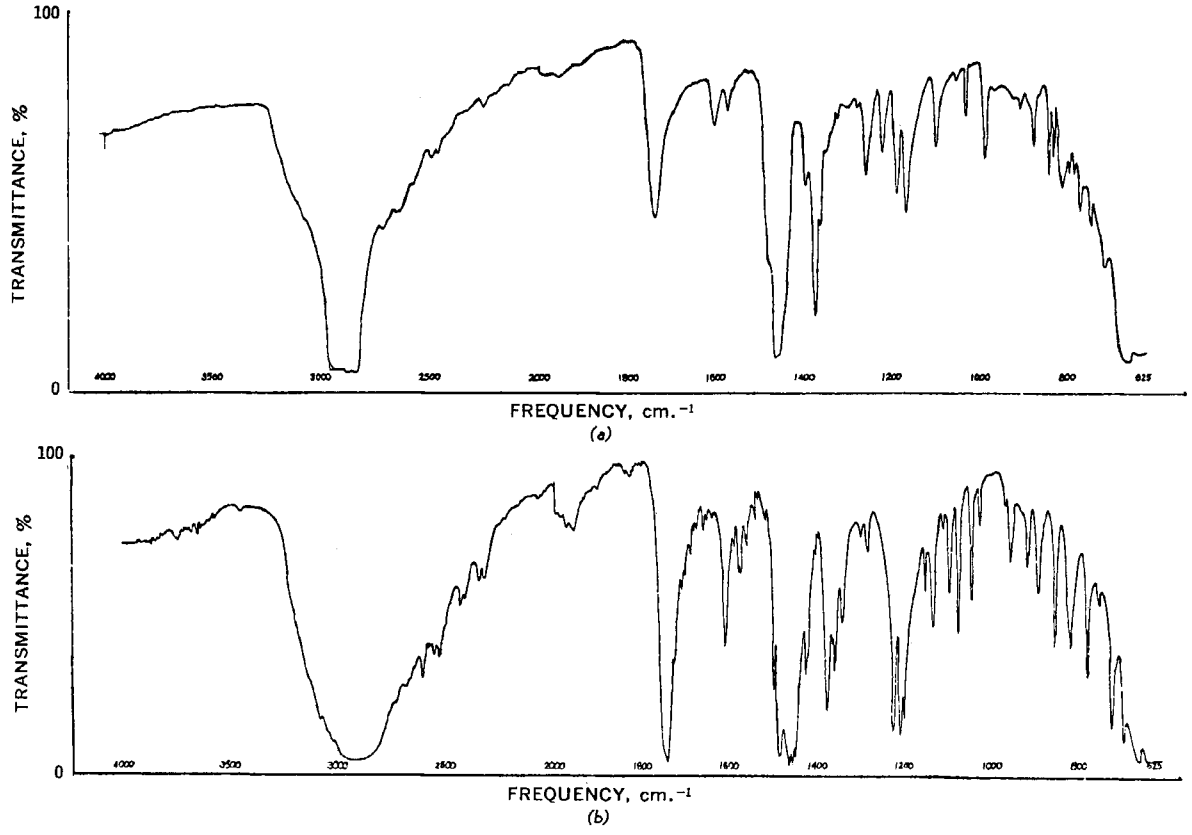


**Figure 1**—*IR spectra of:* (a) L-*protio-phenylalanine hydrochloride, and* (b) L-*deuterio-phenylalanine hydrochloride.*

The residues from each of the fractions representing L-deuterio-phenylalanine hydrochloride were dissolved in a minimum volume of water, combined, and reevaporated to dryness. A small quantity of the residue was employed for IR analysis. The remainder was converted to the free amino acid by the technique of Hirs *et al.* (10) as described below.

A chromatographic column containing Dowex 1-X8 (110 g.) was washed with 3 *M* sodium acetate solution until the effluent was chloride free, then with distilled water until the effluent left no residue upon evaporation, and finally with 0.5 *M* acetic acid until the effluent was acidic to litmus paper. The residue of L-deuterio-phenylalanine hydrochloride was dissolved in 5 ml. of 0.5 *M* acetic acid and loaded on the column. The amino acid was eluted from the column with 0.5 *M* acetic acid, and the amino acid, contained in the first 20 ml. of effluent, was freed of solvent *in vacuo* at room temperature. The white-gray residue, 420 mg., was then crystallized twice from acetone–water. The yield of purified L-deuterio-phenylalanine was 210 mg., m.p. 262° [lit. (11) m.p. 260–262°]. Using the same melting-point apparatus[2], a known sample of L-protio-phenylalanine melted at 283° [lit. (12) m.p. 283°].

*Anal.*—Calc. for $C_9H_3{}^2H_8NO_2$: C, 62.39; H + $^2$H, 11.06; N, 8.08. Found: C, 61.67; H + $^2$H, 10.27; N, 8.33.

Optical activity of the protio and deuterio forms of L-phenylalanine was determined[3]. Water was employed as a blank in these determinations. The observed specific rotation for L-protio-phenylalanine, $[\alpha]_D^{32}$, was −35.4° (c, 1.94 in water) [lit. (12) $[\alpha]_D^{29}$ −35.5°]. The observed specific rotation for L-deuterio-phenylalanine, $[\alpha]_D^{32}$, was −35.1° (c, 1.94 in water) [lit. (11) $[\alpha]_D^{25}$ −34.0° (c, 1.00)].

Both the protio and deuterio forms of the amino acid were subjected to TLC on silica gel G plates (5 × 20 cm., 250 $\mu$ in thickness) with two different solvent systems. Solvent System A contained isopropyl alcohol–water (19:1) and Solvent System B contained phenol–water (3:1) to which was added 20 mg. of sodium cyanide. The chromatograms were developed to a height of 10 cm. in saturated chromatographic chambers. The chromatograms were air dried after being developed, sprayed with ninhydrin reagent, and then placed in a drying oven at 100° for 10 min. The positions where the first ninhydrin-positive color appeared were marked for determining the $R_f$ values for each form of the amino acid. With Solvent System A, the $R_f$ values for the protio and deuterio species were 0.30 and 0.28, respectively; for Solvent System B, the values were 0.62 and 0.59, respectively.

The IR spectra of L-protio-phenylalanine hydrochloride and L-deuterio-phenylalanine hydrochloride were determined with an IR spectrophotometer[4] as mineral oil mulls between two plates[5]. Spectra of the protio and deuterio analogs of the amino acid hydrochloride were compared with a standard reference spectrum of the protio compound. All three spectra showed peaks at 2900 (—$NH_3^+$-$Cl^-$), 1750 (—C=O), 1450 (aromatic nucleus), and 1600 (—$NH_3^+$) cm.$^{-1}$. At 1380 cm.$^{-1}$, the peak pattern and intensity differed for the protio and deuterio species. The spectrum of the deuterio form showed a multiple signal at 1150–1200 cm.$^{-1}$ and a single sharp peak at 990 cm.$^{-1}$, both of which were absent in the spectrum of the protio form. The spectra are shown in Fig. 1.

The NMR spectra of both forms were obtained on an NMR spectrometer[6] as saturated solutions in deuterium oxide, using an internal tetramethylsilane standard. The spectrum for L-protio-phenylalanine showed a multiplet at $\delta$ 3.15–3.35, a quadruplet at $\delta$ 3.95–4.20, a single large peak at $\delta$ 7.50, and a peak at $\delta$ 4.85. The spectrum of L-deuterio-phenylalanine showed a single peak at $\delta$ 4.85 only.

The mass spectra for the protio and deuterio analogs were obtained with a mass spectrometer[7]. The spectrum of L-protio-phenylalanine showed the largest fragment at *m/e* 165 and three high percentage fragments at *m/e* 120, 91, and 74. The spectrum of L-deuterio-phenylalanine showed the largest fragment at *m/e* 173 and three high percentage fragments at *m/e* 128, 98, and 75.

**Transamination Study**—The following reagents were used in this study: potassium phosphate buffer, 0.10 *M*, pH 7.0; potassium phosphate buffer, 0.40 *M*, pH 8.0; arsenate–borate reagent solution, 1.00 *M*, pH adjusted to 6.5 with concentrated hydrochloric acid; L-protio-phenylalanine stock solution, 0.10 *M* in potassium phosphate buffer, 0.40 *M*, pH 8.0; L-deuterio-phenylalanine stock solu-

[2] Thiele.
[3] In 1.6 × 100-mm. Koehler polarimeter cells with a Rudolph polarimeter, model 80.
[4] Perkin-Elmer model 257.
[5] Irtran 2.
[6] Varian A-60.
[7] Hitachi-Perkin-Elmer RMU-6D.

Table I—Reaction Mixtures for the Enzymatic Transamination between L-Protio-phenylalanine and α-Ketoglutarate[a]

| Set | Reaction | 0.10 *M* L-Protio-phenylalanine, ml. | 0.02 *M* Pyridoxal-5-phosphate, ml. | 0.20 *M* α-Keto-glutarate, ml. | Enzyme Solution[b], ml. | Buffer Solution[c], ml. |
|---|---|---|---|---|---|---|
| A | Blank | 0.45 | 0.01 | — | 1.0 | 1.55 |
| | 1 | 0.45 | 0.01 | 0.25 | 1.0 | 1.30 |
| | 2 | 0.45 | 0.01 | 0.15 | 1.0 | 1.40 |
| | 3 | 0.45 | 0.01 | 0.10 | 1.0 | 1.45 |
| | 4 | 0.45 | 0.01 | 0.05 | 1.0 | 1.50 |
| B | Blank | 0.25 | 0.01 | — | 1.0 | 1.75 |
| | 5 | 0.25 | 0.01 | 0.25 | 1.0 | 1.50 |
| | 6 | 0.25 | 0.01 | 0.15 | 1.0 | 1.60 |
| | 7 | 0.25 | 0.01 | 0.10 | 1.0 | 1.65 |
| | 8 | 0.25 | 0.01 | 0.05 | 1.0 | 1.70 |
| C | Blank | 0.20 | 0.01 | — | 1.0 | 1.80 |
| | 9 | 0.20 | 0.01 | 0.25 | 1.0 | 1.55 |
| | 10 | 0.20 | 0.01 | 0.15 | 1.0 | 1.65 |
| | 11 | 0.20 | 0.01 | 0.10 | 1.0 | 1.70 |
| | 12 | 0.20 | 0.01 | 0.05 | 1.0 | 1.75 |
| D | Blank | 0.10 | 0.01 | — | 1.0 | 1.90 |
| | 13 | 0.10 | 0.01 | 0.25 | 1.0 | 1.65 |
| | 14 | 0.10 | 0.01 | 0.15 | 1.0 | 1.75 |
| | 15 | 0.10 | 0.01 | 0.05 | 1.0 | 1.85 |

[a] A total of 12 measurements was performed upon four different concentrations of L-deuterio-phenylalanine in combination with three different concentrations of α-ketoglutarate. (The lowest concentration was excluded from Sets A, B, and C.) [b] One milliliter equivalent to 300 mcg. of protein. [c] Potassium phosphate, 0.40 *M*, pH 8.0.

tion, 0.10 *M*, in potassium phosphate buffer, 0.40 *M*, pH 8.0; α-ketoglutarate stock solution, 0.20 *M* in potassium phosphate buffer, 0.40 *M*, pH 8.0; pyridoxal-5-phosphate stock solution, 0.02 *M*, stored in amber bottle; sodium phenylpyruvate stock solution, freshly prepared, 0.05 *M* in potassium phosphate buffer, 0.40 *M*, pH 8.0; and transamination enzyme stock solution[8], purified rat brain extract (acetone powder, dried) (used as the source of the enzyme system for these studies).

The protein concentration of the rat brain extract was analyzed according to the method of Lowry *et al.* (13), using protein standard solution[9] as a reference standard. The stable enzyme solution preparation was obtained in the following manner. One gram of purified rat brain extract was added to 60 ml. of 0.10 *M* potassium phosphate (pH 7.0) previously cooled to 4°. The mixture was then stirred at this temperature for 45 min. The clear supernate obtained by decantation and subsequent filtration was used.

With the exception of the arsenate–borate stock solution (stored at 0°) and sodium pyruvate stock solution (freshly prepared), all other stock solutions were stored at −20°.

A standard calibration curve for determining the pyruvate concentration was prepared by using the sodium phenylpyruvate stock solution (0.05 *M*) as a reference standard. Varying amounts (50–500 nmoles) were added to a basic reaction mixture containing α-ketoglutarate (50 μ*M*, pH 8.0), pyridoxal-5-phosphate (200 n*M*), potassium phosphate buffer (555 μ*M*, pH 8.0), and enzyme (equivalent to 300 mcg. of protein) in a final volume of 3.1 ml. The mixture was incubated at 37° for 15 min. The rate of transamination was followed by measuring the absorbance of the enol–borate complex formed from the phenylpyruvate generated during the incubation (14) at 300 nm., using a spectrophotometer[10]. The arsenate-catalyzed modification described by George *et al.* (7) was applied to the assay procedure.

The standard reaction mixture contained: potassium phosphate buffer (555 μ*M*, pH 8.0), α-ketoglutarate (10–50 μ*M*, added last), pyridoxal-5-phosphate (200 n*M*), L-protio- or L-deuterio-phenylalanine (10–45 μ*M*), and enzyme (rat brain extract, equivalent to 300 mcg. of protein) in a final volume of 3.1 ml. The α-ketoglutarate was omitted in the blank. The reaction mixture minus the α-ketoglutarate was preincubated at 37° in a constant-temperature bath for 10 min. Transamination was then generated by the addition of the α-ketoglutarate to the reaction mixture. An amount of potassium phosphate buffer (pH 8.0) equivalent to the amount of α-ketoglutarate added to the reaction mixture was then added to the blank.

An aliquot (0.5 ml.) was withdrawn from both the reaction mixture and reaction blank (free of α-ketoglutarate), and each was added to separate solutions of trichloroacetic acid (0.25 ml., 25% w/v) at appropriate time intervals (1–10 min.).

The precipitated enzyme protein was removed by centrifugation, after which a 0.5-ml. aliquot of the reaction mixture supernate was removed and added to 2.5 ml. of arsenate–borate reagent. The thoroughly mixed solution was then allowed to stand at room temperature for 20 min. A sample of the reaction blank was treated similarly. The absorbance of the sample relative to the blank was measured at 300 nm.

**Kinetic Measurements**—A total of 15 measurements was performed on four different concentrations of L-protio-phenylalanine in combination with three to four different concentrations of α-ketoglutarate. A similar series of 12 measurements was conducted with the deuterio analog in combination with three concentrations

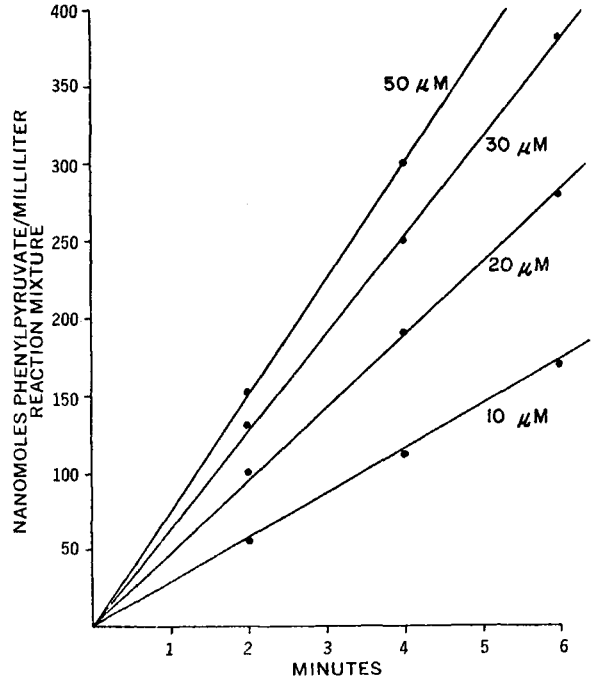


**Figure 2**—*Rate of transamination between* L-*protio-phenylalanine and* α-*ketoglutarate* (L-*protio-phenylalanine, 45* μM; *0.40* M *phosphate buffer, pH 8.0; 37°; pyridoxal-5-phosphate, 200* n*M; enzyme concentration, 300 mcg./ml.).*

[8] Obtained from Schwarz-Mann Research Laboratories, Inc.
[9] Bovine serum albumin, Armour & Co.
[10] Zeiss PMQ II.

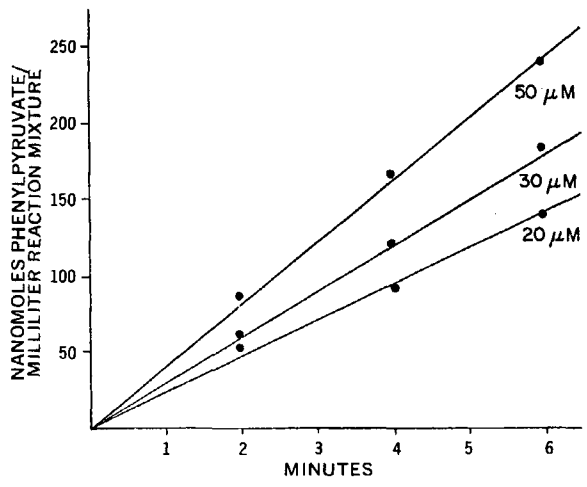


**Figure 3**—*Rate of transamination between* L-*deuterio-phenylalanine and α-ketoglutarate* (L-*deuterio-phenylalanine, 45 μM; 0.40* M *phosphate buffer, pH 8.0; 37°; pyridoxal-5-phosphate, 200 n*M; *enzyme concentration, 300 mcg./ml.*).

of α-ketoglutarate. The quantities of all reactants employed in these determinations are given in Table I. Graphical presentations of the initial velocities obtained are given in Figs. 2 and 3 for the protio and deuterio analogs, respectively, at a concentration level of 45 μ*M*. The initial velocities obtained from these determinations and their respective reciprocal values are given in Tables II and III.

## DISCUSSION

**Isolation of** L-**Deuterio-phenylalanine**—The technique of continuous gradient elution was applied to the final step of the procedure to isolate L-deuterio-phenylalanine. Since the mixture containing the desired amino acid had been stripped of the deuterated acidic amino acids, L-aspartic and L-glutamic acids, a polystyrene sulfonic acid resin was chosen because of its ability to separate the basic amino acids from the neutral amino acids. The use of conventional elution techniques to separate the individual neutral amino acids by ion-exchange chromatography is not possible. For example, phenylalanine is usually contaminated with leucine. Although the technique of continuous gradient elution can be effectively used for separating compounds with similar chromatographic properties, it is successful only if the proper gradient increase in the concentration of the eluting agent is used.

L-Protio-leucine was selected as a contaminant of L-protio-phenylalanine for determining the gradient change in hydrochloric acid concentration required for effecting separation because it is

**Table II**—Initial Velocities of Transamination Reactions between L-Phenylalanine and α-Ketoglutarate (Enzyme Concentration, 300 mcg./ml.; 37°; pH 8.0; Pyridoxal-5-phosphate, 200 n*M*; Phosphate Buffer, 0.4 *M*)

| Reaction | L-Phenylalanine, μ*M* | α-Ketoglutarate, μ*M* | Initial Velocity ($V_i$), n*M*/ml./min. | $1/V_i$ |
|---|---|---|---|---|
| 1 | 45.00 | 50.00 | 75.00 | 0.0133 |
| 2 | 45.00 | 30.00 | 63.00 | 0.0159 |
| 3 | 45.00 | 20.00 | 46.75 | 0.0214 |
| 4 | 45.00 | 10.00 | 29.25 | 0.0342 |
| 5 | 25.00 | 50.00 | 63.75 | 0.0157 |
| 6 | 25.00 | 30.00 | 50.75 | 0.0197 |
| 7 | 25.00 | 20.00 | 41.25 | 0.0242 |
| 8 | 25.00 | 10.00 | 27.50 | 0.0364 |
| 9 | 20.00 | 50.00 | 56.25 | 0.0177 |
| 10 | 20.00 | 30.00 | 45.00 | 0.0222 |
| 11 | 20.00 | 20.00 | 37.50 | 0.0266 |
| 12 | 20.00 | 10.00 | 26.25 | 0.0381 |
| 13 | 10.00 | 50.00 | 45.75 | 0.0219 |
| 14 | 10.00 | 30.00 | 38.75 | 0.0258 |
| 15 | 10.00 | 10.00 | 22.00 | 0.0454 |

**Table III**—Initial Velocities of Transamination Reactions between L-Deuterio-phenylalanine and α-Ketoglutarate (Enzyme Concentration, 300 mcg./ml.; 37°; pH 8.0; Pyridoxal-5-phosphate, 200 n*M*; Phosphate Buffer, 0.4 *M*)

| Reaction | L-Deuterio-phenylalanine, μ*M* | α-Ketoglutarate, μ*M* | Initial Velocity ($V_i$), n*M*/ml./min. | $1/V_i$ |
|---|---|---|---|---|
| 1 | 45.00 | 50.00 | 40.00 | 0.0250 |
| 2 | 45.00 | 30.00 | 30.00 | 0.0333 |
| 3 | 45.00 | 20.00 | 23.00 | 0.0435 |
| 4 | 25.00 | 50.00 | 35.75 | 0.0280 |
| 5 | 25.00 | 30.00 | 27.00 | 0.0370 |
| 6 | 25.00 | 20.00 | 21.25 | 0.0471 |
| 7 | 20.00 | 50.00 | 29.25 | 0.0342 |
| 8 | 20.00 | 30.00 | 23.25 | 0.0430 |
| 9 | 20.00 | 20.00 | 20.00 | 0.0500 |
| 10 | 10.00 | 50.00 | 23.25 | 0.0430 |
| 11 | 10.00 | 30.00 | 18.75 | 0.0533 |
| 12 | 10.00 | 10.00 | 11.25 | 0.0899 |

the amino acid most difficult to separate from L-phenylalanine. When using a gradient of 1–4 *N* HCl, the elution volumes for the protio forms of L-leucine and L-phenylalanine were 1655–1990 and 3235–3750 ml., respectively. With the deuterio forms, the elution volumes for L-leucine and L-phenylalanine were 1550–1850 and 2880–3600 ml., respectively.

**Identification and Characterization**—L-Deuterio-phenylalanine was identified on the basis of its melting point, elemental analysis, specific rotation, and a comparison of its $R_f$ value with L-protio-phenylalanine in two solvent systems using TLC. For the last, lower $R_f$ values were obtained for the deuterio compound. This finding is in agreement with earlier studies (2, 9) involving other deuterio amino acids.

The IR spectrum of the deuterio compound showed excellent correlation with the protio amino acid, particularly in the higher regions (3500–1450 $cm.^{-1}$). Marked differences were noted in the "fingerprint" region of the spectrum (1400–900 $cm.^{-1}$). The spectrum for the deuterio amino acid showed a multiple signal at 1200–1150 $cm.^{-1}$ and a single sharp peak at 990 $cm.^{-1}$. These peaks were absent in the spectrum of the protio derivative. Generally, the peaks in the fingerprint region resulted from bending and stretching vibrations of C—O, C—N, and C—H bonds.

The NMR spectra were compared in a spectrometer[6]. The spectrum for the protio compound revealed the nonequivalence of the protons on the α- and β-carbons and the equivalency of the protons of the aromatic nucleus. The peak observed at δ 4.85 is due to water formed by exchange at the amino and carboxyl functional groups. Integration of the spectral peaks confirmed the peak assignments. The spectrum for L-deuterio-phenylalanine revealed the presence of a lone water peak. The absence of proton signals in the spectrum indicated that nonexchangeable sites in the molecule were fully deuterated.

The mass spectra of L-protio- and L-deuterio-phenylalanine showed the largest fragments at *m/e* 165 and 173, respectively, which correspond to their respective molecular weights. The low percentage of the two fragments can be attributed to the low volatility of the amino acids in their zwitterion form (15). Cleavage of the carboxyl group from the protio species yields $C_6H_5—CH_2—{}^+CH—NH_2$ as a fragment, with a molecular weight of 120. The same type of cleavage from the deuterio species yields $C_6D_5—CD_2—{}^+CD—NH_2$ as a fragment, with a molecular weight of 128.

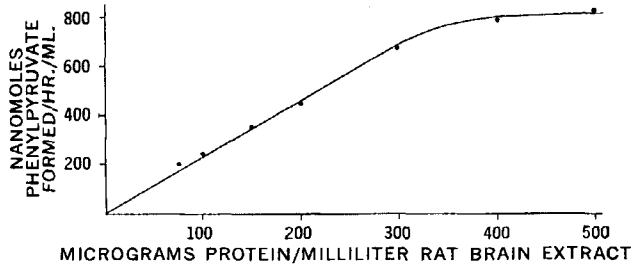


**Figure 4**—*Effect of enzyme concentration on the* in vitro *formation of phenylpyruvate.*

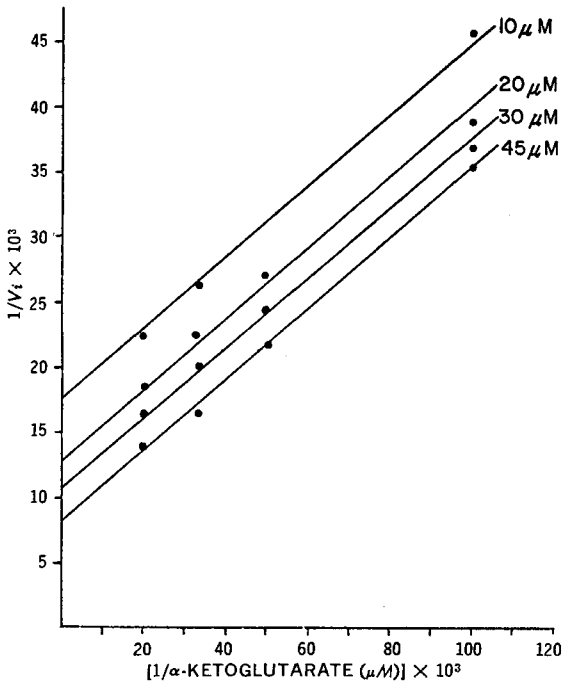


**Figure 5**—*Double reciprocal plot of initial velocities* versus *α-keto-glutarate concentrations at a series of fixed concenirations of* L-*protio-phenylalanine.*

The high percentage fragments appearing at *m/e* 74 and 91 in the protio species spectrum are attributed to bond cleavage between the α- and β-carbons, *e.g.*, $C_6H_5$—$^+C_\beta H_2$ (mol. wt. 91) and $^+CH(NH_2)$-COOH (mol. wt. 74). Similar cleavage, as observed in the deuterio spectrum, gives rise to the fragmentation peaks observed at *m/e* 98 and 75, respectively, which correspond to the molecular weights of fragments $C_6D_5$—$^+C_\beta D_2$ (mol. wt. 98) and $^+CD(NH_2)COOH$ (mol. wt. 75).

**Transamination Studies**—The rate of L-protio-phenylalanine metabolism was determined by analysis of the reaction for phenyl-pyruvate at regular time intervals while samples were incubated at 37° for 2 hr. The reaction was found to proceed linearly over the first 6–8 min., after which the rate of formation of phenylpyruvate decreased. This decrease in phenylpyruvate formation may be attributed to inactivation of the enzyme and/or breakdown of the phenylpyruvate formed to phenylacetaldehyde or phenylacetate.

To establish the enzyme concentration range over which the observed initial velocity is directly proportional to the enzyme concentration, L-protio-phenylalanine (45 μ*M*) was incubated in the standard reaction mixture (containing 30 μ*M* of α-ketoglutarate) with varied amounts of rat brain extract. The results are shown in Fig. 4.

The steady-state kinetics of transamination may be represented by the following equation:

$$\frac{1}{V_i} = \frac{1}{V_{max}\cdot[E]}\left(1 + \frac{K_{Am}}{[Am]} + \frac{K_{Ket}}{[Ket]}\right) \qquad \text{(Eq. 1)}$$

where $V_i$ = initial velocity, $V_{max}$ = maximum velocity, $[E]$ = enzyme concentration, $K_{Am}$ = Michaelis constant for the amino acid, $K_{Ket}$ = Michaelis constant for the keto acid, $[Am]$ = amino acid concentration, and $[Ket]$ = keto acid concentration.

Under the assumptions of steady-state kinetics, the initial velocity, $V_i$, of an enzymatic reaction will increase with an increase in the concentration of substrates.

Double reciprocal plots of determined initial velocities, $1/V_i$ (Tables II and III), *versus* α-ketoglutarate concentrations at a series of fixed concentrations of L-protio-phenylalanine or L-deuterio-

**Table IV**—Michaelis Constants and Maximum Velocities Obtained from the Transamination Reactions between α-Ketoglutarate and L-Phenylalanine Analogs (0.4 *M* Phosphate Buffer; pH 8.0; 37°; Pyridoxal-5-phosphate, 200 n*M;* Enzyme Concentration, 300 mcg./ml.)

| Amino Acid Substrate | $K_{Am}$[a], μ*M* | $K_{Ket}$[b], μ*M* | $V_{max}\cdot[E]$[c] | $V_H/V_D$ |
|---|---|---|---|---|
| L-Protio-phenylalanine | 21.33 | 45.77 | 0.1742 | 1.00 |
| L-Deuterio-phenylalanine | 26.19 | 65.23 | 0.1062 | 1.64 |

[a] Michaelis constants of the amino acids. [b] Michaelis constants of α-ketoglutarate. [c] Maximum velocities at the enzyme concentration (300 mcg./ml.).

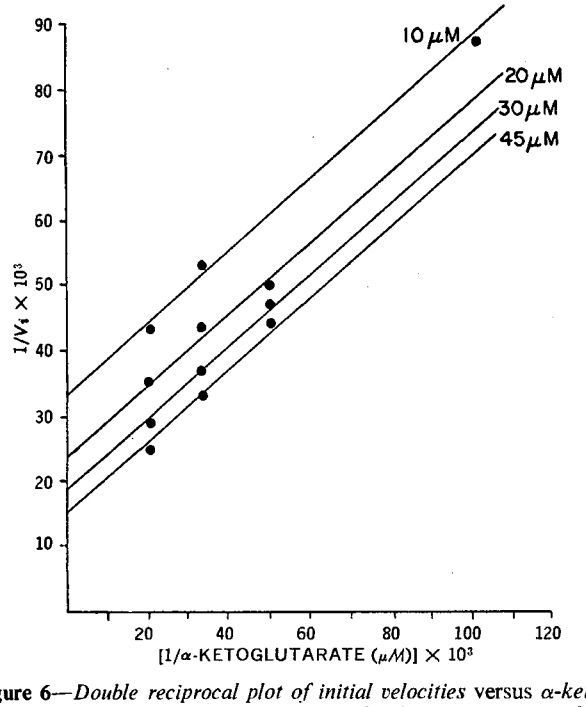


**Figure 6**—*Double reciprocal plot of initial velocities* versus *α-keto-glutarate concentrations at a series of fixed concentrations of* L-*deuterio-phenylalanine.*

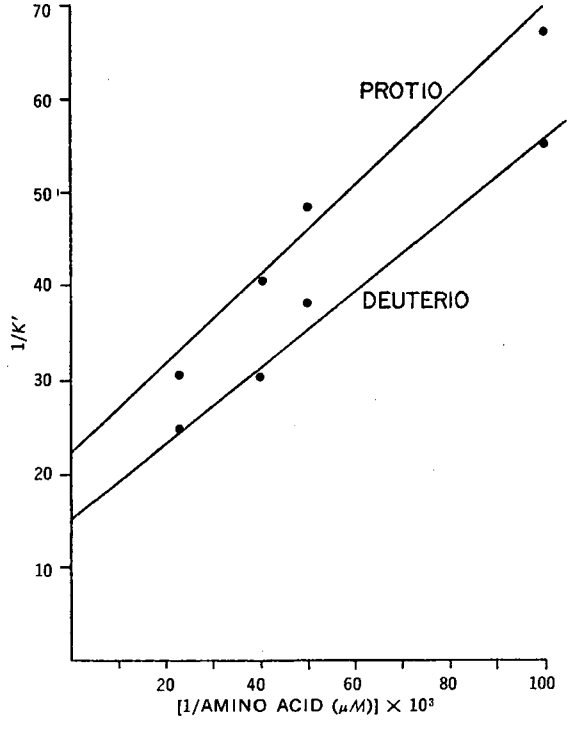


**Figure 7**—*Double reciprocal plot of apparent Michaelis constants* versus *amino acid concentrations of* L-*phenylalanine analogs.*

Table V—Intermediate Values of the Statistical Analyses on the Kinetic Studies of the Transamination Reactions between α-Ketoglutarate and L-Phenylalanine Analogs

| Amino Acid Substrate | $S_1$[a], min./m$M$/ml. | $I_1$, min./n$M$/ml. | $1/K'$, $\mu M^{-1}$ | $S_2$ | $I_2$, $\mu M^{-1}$ | $S_3$, min./m$M$/ml. | $I_3$, min./n$M$/ml. |
|---|---|---|---|---|---|---|---|
| L-Protio-phenylalanine | | | | 0.4660 | 21.85 | 0.1225 | 5.74 |
| 45 $\mu M$ | 0.2628 | 7.86 | 29.91 | | | | |
| 25 $\mu M$ | 0.2628 | 10.66 | 40.56 | | | | |
| 20 $\mu M$ | 0.2628 | 12.81 | 48.74 | | | | |
| 10 $\mu M$ | 0.2628 | 17.60 | 66.97 | | | | |
| L-Deuterio-phenylalanine | | | | 0.4015 | 15.33 | 0.2344 | 9.42 |
| 45 $\mu M$ | 0.5837 | 13.88 | 23.78 | | | | |
| 25 $\mu M$ | 0.5837 | 17.28 | 29.60 | | | | |
| 20 $\mu M$ | 0.5837 | 22.38 | 38.34 | | | | |
| 10 $\mu M$ | 0.5837 | 31.93 | 54.70 | | | | |

[a] The slope given here is the average value.

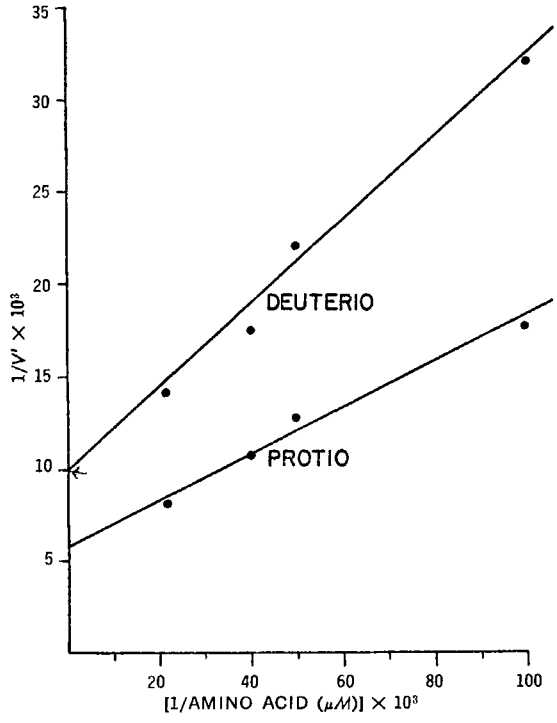


**Figure 8**—*Double reciprocal plot of apparent maximum velocities* versus *amino acid concentrations of* L-*phenylalanine analogs.*

phenylalanine are shown in Figs. 5 and 6, respectively. The $y$-intercept, $I_1$, of each plot yields the reciprocal of the apparent maximum velocity, $1/V'$. The corresponding slope, $S_1$, yields the ratio of the Michaelis constant for the keto acid, $K_{Ket}$, to the product of the maximum velocity and enzyme concentration, $V_{max}[E]$. The ratio of the slope of the plot, $S_1$, to the corresponding $y$-intercept, $I_1$, is defined as the apparent Michaelis constant, $K'$.

Double reciprocal plots of apparent Michaelis constants, $1/K'$, *versus* amino acid concentrations, $1/[Am]$, of the protio and deuterio analogs of L-phenylalanine are given in Fig. 7. The $y$-intercept of these plots, $I_2$, yields the reciprocal of the Michaelis constant of the keto acid, $1/K_{Ket}$. The corresponding slope, $S_2$, of these plots is equal to the ratio of the Michaelis constant of the amino acid (protio or deuterio analog) to the Michaelis constant of the keto acid, $K_{Am}/K_{Ket}$.

The maximum velocity, $V_{max}$, for the protio and deuterio forms of L-phenylalanine was determined, as shown in Fig. 8, by means of a double reciprocal plot of apparent maximum velocities, $1/V'$, *versus* protio and deuterio amino acid concentration, $1/[Am]$. The $y$-intercept of these plots yields the reciprocal of the maximum velocity, $1/V_{max}$, for the corresponding analogs.

The values of $K_{Am}$, $K_{Ket}$, and $V_{max}[E]$ (Table IV) were obtained by statistical analysis of the experimental data. Least-squares regression analysis was employed to obtain the equations representing the best-fit straight lines in Figs. 5 and 6. The slopes, $S_1$, and the intercepts, $I_1$, obtained by statistical derivation were subsequently used to determine the best-fit straight lines shown in Fig. 7. The best-fit straight lines for those lines shown in Fig. 8 were also obtained by statistical analysis. Table V shows the intermediate values obtained from the statistical analysis.

The maximum velocity for L-deuterio-phenylalanine was found to be 61% of the value obtained for the protio analog.

The deuterium isotope effect, expressed as the $V_H/V_D$ value (the ratio of the maximum velocity of L-protio-phenylalanine to that of L-deuterio-phenylalanine), was found to be 1.64.

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