Proc. Nat. Acad. Sci. USA
Vol. 69, No. 5, pp. 1086–1090, May 1972

Reversibility of the Tryptophanase Reaction: Synthesis of Tryptophan from Indole, Pyruvate, and Ammonia

(*E. coli*/α-aminoacrylate/Michaelis–Menten kinetics/pyridoxal 5′-phosphate)

TAKEHIKO WATANABE AND ESMOND E. SNELL

Department of Biochemistry, University of California, Berkeley, Calif. 94720

Contributed by Esmond E. Snell, February 14, 1972

ABSTRACT **Degradation of tryptophan to indole, pyruvate, and ammonia by tryptophanase (EC 4....) from *Escherichia coli*, previously thought to be an irreversible reaction, is readily reversible at high concentrations of pyruvate and ammonia. Tryptophan and certain of its analogues, e.g., 5-hydroxytryptophan, can be synthesized by this reaction from pyruvate, ammonia, and indole or an appropriate derivative at maximum velocities approaching those of the degradative reactions. Concentrations of ammonia required for the synthetic reactions produce specific changes in the spectrum of tryptophanase that differ from those produced by K^+ and indicate that ammonia interacts with bound pyridoxal 5′-phosphate to form an imine. Kinetic results indicate that pyruvate is the second substrate bound, hence indole must be the third. These results favor a modified mechanism for the multitude of tryptophanase-catalyzed reactions in which α-aminoacrylate, which functions as a common enzyme-bound intermediate in both synthetic and degradative reactions, is not released into the medium during the latter reactions, but is degraded to pyruvate and ammonia by sequential reversible steps via enzyme-bound intermediates.**

Tryptophanase (E.C.4....)* was originally shown to catalyze reaction **1** by Wood *et al.* (1). Although in subsequent studies it was generally considered to catalyze only the degradation of tryptophan and certain of its analogues, more recent investigations with the pure enzyme (2, 3) showed that at higher substrate concentrations, tryptophanase catalyzes many other α,β-elimination reactions, shown in Eq. **2**, as well as numerous β-replacement reactions (Eq. **3**).

$$\text{Tryptophan} + H_2O \rightarrow \text{Indole} + \text{Pyruvate} + NH_3 \quad (1)$$

$$RCH_2CHNH_2COOH + H_2O \rightarrow RH + CH_3COCOOH + NH_3 \quad (2)$$

$$RCH_2CHNH_2COOH + R'H \rightarrow RH + R'CH_2CHNH_2COOH \quad (3)$$

In equations **2** and **3**, *R* may represent -OH, -SH, $-OCH_3$, $-SCH_3$, various substituted indolyl radicals, or other appropriate electronegative groups, and R′H represents indole or certain substituted indoles. Reactions **1–3** were shown (4)† to proceed through a common intermediate, probably an enzyme-bound α-aminoacrylic acid, which could either decompose to pyruvate and ammonia (in reactions **1** and **2**) or add indole to form tryptophan (in reaction **3**). At concentrations previously tested, reactions **1** and **2** were irreversible (4).

Subsequent to these investigations, Yamada *et al.* (5–7) showed that β-tyrosinase from *Escherichia intermedia* catalyzes reaction **4** but not reaction **1**, and is similar in many respects to tryptophanase.

$$\text{Tyrosine} + H_2O \rightarrow \text{Phenol} + \text{Pyruvate} + NH_3 \quad (4)$$

It, too, catalyzes degradation of serine, cysteine, etc. according to reaction **2**, and synthesizes tyrosine from these same amino acids in the presence of phenol (R′H in Eq. **3**), apparently by a reaction mechanism closely analogous to that of tryptophanase. However, β-tyrosinase also synthesizes tyrosine from phenol in the presence of high concentrations of pyruvate and ammonia (8), apparently by reversal of reaction **4**.

Appropriate trials, described herein, have now shown that when the concentrations of pyruvate and NH_3 are raised sufficiently, tryptophanase catalyzes synthesis of tryptophan by reversal of reaction **1** at rates similar to the forward reaction. This reverse reaction provides useful additional information concerning the mechanism of α,β-elimination reactions.

METHODS

Isolation of Tryptophanase. Tryptophanase was purified from *Escherichia coli* B/1t7A according to the published procedure (9) with slight modifications as follow: (*a*) The use of dithiothreitol (0.2 mM) in place of 2-mercaptoethanol as an -SH group protector increased stability of the enzyme, so that final specific activities (in μmol of tryptophan decomposed per mg of enzyme per min) of 24–27 were obtained. (*b*) After the second ammonium sulfate fractionation of the published procedure, the enzyme solution was dialyzed against 0.06 M potassium phosphate buffer (pH 7.75) containing 1 mM EDTA–0.1 mM dithiothreitol–0.04 mM pyridoxal 5′-phos-

* This enzyme, depending upon the substrate chosen, acts as a lyase cleaving carbon–carbon, carbon–oxygen, carbon–sulfur, or carbon–nitrogen bonds. No Enzyme Commission number has been assigned as yet.

Abbreviation: Pyridoxal-P, pyridoxal 5′-phosphate.

† In the kinetic study cited, an unfortunate error resulted in inversion of one term in the rate equation for case III (corresponding to Eq. **3** in this paper). This error was kindly brought to our attention by Miss (now Dr.) Regina Ziegler, together with the heartening information that it did not change the overall conclusions drawn. In rate Eq. III (in ref. 4), the term $\ldots + k_{-1}Kc'/k_5(C)$ should be inverted.

phate (pyridoxal-P) and applied to a DEAE-cellulose column equilibrated with the same buffer. Tryptophanase was not absorbed to the column under these conditions, but minor components that are removed only with difficulty by subsequent crystallization are absorbed. Active fractions from the column were combined. After repeated precipitation of tryptophanase by 66% saturation with ammonium sulfate in the presence of 10 mM DL-penicillamine, apotryptophanase was crystallized according to the published method (3). 4–5 Recrystallizations were necessary to reach constant specific activity.

Assay of Tryptophanase. The enzyme was assayed routinely in 0.4-ml volumes by measuring the indole formed from tryptophan in reaction 1 by the Ehrlich reaction, as described (10).

Biosynthesis of Tryptophan. Enzymatic synthesis of tryptophan was followed at 37° in 0.5-ml reaction mixtures typically containing, at pH 8.6, 0.1 M *N,N*-bis[2-hydroxyethyl]glycine (Bicine), 0.1 M KCl, 0.08 mM pyridoxal-P, 0.1 mM dithiothreitol, 0.08 mM indole, 0.2 M NH_4Cl, 0.2 M potassium pyruvate, and enzyme. Variations from these conditions are noted in the Figures. Reactions were started by addition of potassium pyruvate or enzyme, and were stopped after 5–15 min by addition of 0.1 ml of 5% KOH. When relatively high concentrations of tryptophan were formed (0.08–0.6 μmol/0.5 ml), the amino acid was determined by Udenfriend's procedure (11). During most of the rate studies reported here, low concentrations (2–20 nmol/0.5 ml) of tryptophan were formed. A procedure for determination of small amounts of tryptophan on a microscale was devised. Excess free indole was removed from the mixture by extraction three times with 1.5 ml of toluene; 0.3 ml of the aqueous residue was then removed, neutralized with 0.1 ml of 2.5% HCl, and overlaid with a little toluene. Excess tryptophanase (1 IU, about 40 μg of pure enzyme) was then added to decompose tryptophan in the mixture quantitatively to indole. After 15 min at 37°, indole was estimated as in the direct assay for tryptophanase (see preceding section), but with use of the expanded scale of the Gilford Spectrophotometer. A standard curve (Fig. 1) shows satisfactory recovery of tryptophan added to reaction mixtures.

Other Procedures. Analyses for amino acids and pyridoxamine or its derivatives were made on an automatic amino-acid analyzer (Beckman model 120C) equipped with an expanded scale. Absorption spectra were recorded on the Cary model 14 spectrophotometer.

RESULTS

Tryptophanase-catalyzed synthesis of tryptophan from indole, pyruvate, and ammonia‡

Data of Fig. 2 show that tryptophanase does catalyze the synthesis of tryptophan by reversal of reaction 1 when relatively high concentrations of pyruvate and NH_4Cl are present. Under the conditions used, synthesis is proportional to both time and enzyme concentration. That the product synthesized is L-tryptophan is shown by (*a*) its complete degradation by tryptophanase to yield indole, (*b*) the fact that it migrates identically with authentic tryptophan on paper chromatograms (developing solvent, *n*-butanol:pyridine:acetic acid:water, 15:10:3:12) to give a ninhydrin and Ehrlich-positive spot with R_F = 0.55, and (*c*) that it gives a ninhydrin peak on the amino-acid analyzer at the same position as authentic tryptophan, which was eluted from the short column after 38 min at pH 5.28 and 56.5°.

When 5-hydroxyindole was substituted for indole in reaction mixtures similar to that of Fig. 2, 5-hydroxytryptophan was formed, as shown by paper chromatography (R_F = 0.43 in the solvent described earlier), elution time on the amino-acid analyzer (30 min), and decomposition to 5-hydroxyindole by tryptophanase. In each of these respects the product behaved identically with authentic 5-hydroxy-L-tryptophan.

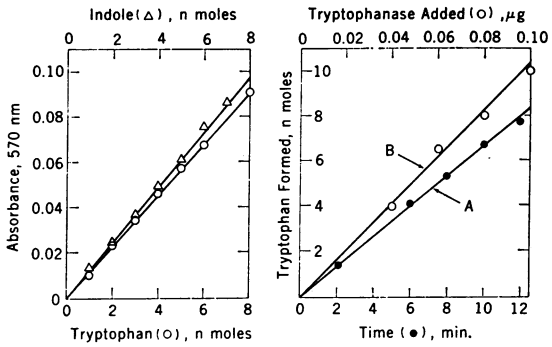

FIG. 1 (*left*). Comparative color formation in the Ehrlich reaction with added free indole (△) or with tryptophan (○) after its conversion to indole with tryptophanase (see *text*). Reaction mixtures were those described for the microdetermination of tryptophan in the text, but indole and tryptophan were varied as indicated.

FIG. 2 (*right*). Tryptophan synthesis from indole, pyruvate, and ammonia as a function of time (*curve A*) and tryptophanase concentration (*curve B*). Reaction mixtures are described in the text. For *curve A*, each reaction vessel contained 40 ng of tryptophanase; the incubation time in establishing *curve B* was 5 min.

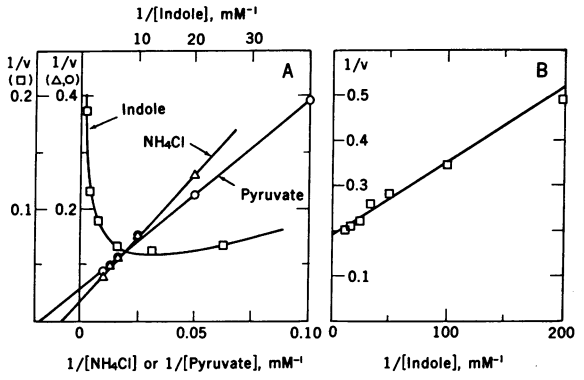

FIG. 3. Relation of rate of tryptophan synthesis (*v*, in nmol per 5 min) to concentration of indole (□), pyruvate (○), or ammonia (△). Tryptophan was determined by the microprocedure after 5 min of incubation at 37°. Tryptophanase per reaction vessel was 145 ng in *A*, and 58 ng in *B*. Concentrations of substrates other than that varied were: pyruvate, 0.2 M; NH_4Cl, 0.2 M; indole, 0.08 mM.

‡ In the ensuing discussion, ammonia and NH_4Cl are used interchangeably to denote the equilibrium mixture of NH_3 and NH_4^+ present at the pH of the reaction mixture.

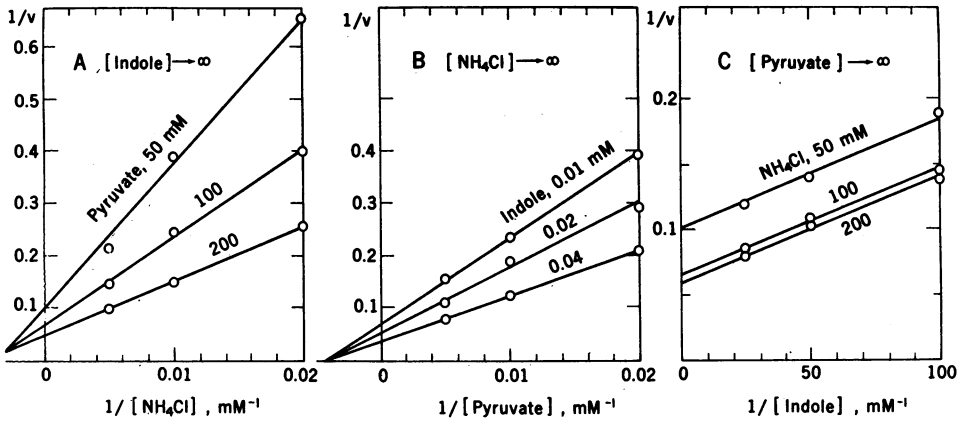

FIG. 4. Kinetic attempts to establish the order of addition of indole, pyruvate, and ammonia to tryptophanase during synthesis of tryptophan. Maximum extrapolated velocities of tryptophan synthesis corresponding to velocities at "infinite concentration" of one substrate, were determined at several fixed concentrations of the two other substrates, from plots such as those of Fig. 3. These maximum velocities (v, in nmol per 5 min) are plotted here (as reciprocals) against the reciprocals of the fixed concentrations of the other two substrates used in their determination (see ref. 12 for theory). Procedures were similar to those described in Fig. 3; each reaction mixture contained 96.5 ng of tryptophanase and was incubated 5 min.

Kinetics of tryptophan synthesis from indole, pyruvate, and ammonia

a. Relation to Substrate Concentration and pH. The velocity of tryptophan synthesis as a function of concentration of indole, pyruvate, and ammonium chloride is shown in Fig. 3. Michaelis–Menten kinetics are observed with pyruvate and ammonia, but indole shows strong substrate inhibition at high concentrations (Fig. 3*A*). The apparent K_m values for indole, pyruvate, and ammonia calculated from Fig. 3 are 0.0085, 55, and 125 mM, respectively; by use of three substrate kinetics (see Fig. 4), more accurate K_m values of 0.019, 70, and 345 mM, respectively, were obtained. The K_m value for tryptophan in the degradative tryptophanase reaction (reaction 1), by contrast, is 0.33 mM (3). The very low affinities for pyruvate and ammonia suffice to explain why synthesis of tryptophan by the reversal of reaction 1 was not observed earlier. In addition, the pH optimum of the synthetic reaction is higher by about 0.5 pH units than that for the degradative reaction (Fig. 5). The maximum velocity of tryptophan biosynthesis at 37°, calculated from data of Fig. 4, is about 19 μmol/mg of enzyme per min, which is very close to the maximum velocity of tryptophan degradation by reaction 1 at 37° (26 μmol/mg of enzyme per min, ref. 3).

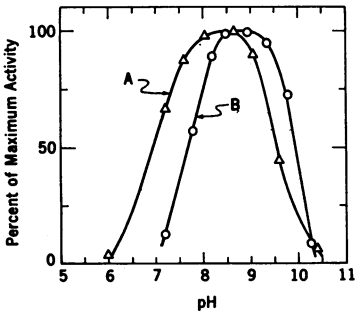

FIG. 5. Comparative pH dependency for the degradation (*curve A*) and synthesis (*curve B*) of tryptophan by tryptophanase. Buffers (0.1 M) were 2-(*N*-morpholino)ethane sulfonic acid (MES) (pH 5.9–7.2), *N,N*-bis[2-hydroxyethyl]glycine (Bicine) (pH 7.6–9.0), or cyclohexylaminopropane sulfonic acid (CAPS) (pH 9.6–10.4). For *curve A*, 6.1 mM tryptophan replaced indole, pyruvate, and NH_4Cl in the standard reaction mixture; 360 ng of tryptophanase was used per vessel, and incubation was for 5 min. For *curve B*, the standard reaction mixture was used with 145 ng of tryptophanase per vessel and 5 min of reaction time.

b. Kinetic Mechanism of the Reaction. Double-reciprocal plots of the extrapolated reaction velocity at infinite concentrations of one substrate and variable concentrations of the remaining two substrates (12) are shown in Fig. 4. A parallel set of curves is obtained only when pyruvate is at "infinite" concentration. These sets of reciprocal plots in three-substrate systems are consistent with the ordered Ter-Uni mechanism of Cleland (13), and indicate that pyruvate is the second substrate to combine with the enzyme. The order of combination of ammonia and indole (i.e., whether first or third) with enzyme is not determined. If one assumes, however, that the synthetic reaction is the reverse of the degradative reaction, then previous studies (4) indicate that ammonia should be the first substrate to interact with the enzyme in the synthetic reaction, and indole should be the third. Confirmation of this ordered sequence was obtained by the following experiments.

Effect of High Concentrations of Ammonia on the Spectrum of Tryptophanase. Either K^+ or NH_4^+ is essential for tryptophanase activity (14). Interaction of these ions with the holoenzyme produces internal changes in structure at the active site, as shown by changes in spectrum of the bound coenzyme, pyridoxal-P (15). These spectral effects of the two cations are compared over a large concentration range in Fig. 6. The two cations elicit complex, but generally similar, changes in the spectrum up to concentrations well above those required for maximum activation of reaction 1 (indicated by the *arrows* to the *abscissa* in Fig. 6). Although differences in the distribution of species that absorb at 337 and at 405–415 nm are evident at concentrations below 50 mM, the sum of the absorbances remains constant in the presence of each cation, and neither cation inhibits the degradative tryptophanase reaction at these concentrations.

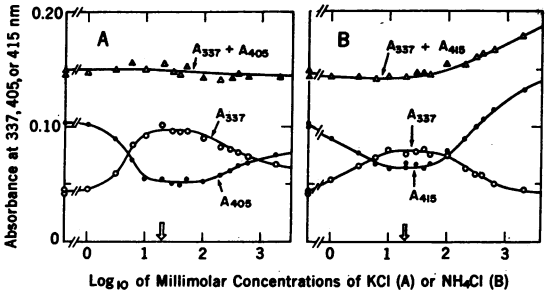

FIG. 6. Effect of KCl (*A*) or NH_4Cl (*B*) on the absorbance of holotryptophanase (1 mg/ml) at 337, 405, or 415 nm. All solutions were in 0.1 mM dithiothreitol–0.1 M triethanolamine buffer (pH 8.5). Apotryptophanase freed of extraneous cations by passage through Sephadex G-25 was converted to holotryptophanase by incubation with exactly one equivalent of pyridoxal-P per subunit (molecular weight 55,000, ref. 15) in the above buffer at pH 7.5 for 15 min before dilution into the cuvettes.

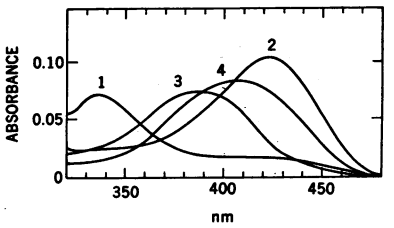

FIG. 7. Spectrum of holotryptophanase (*curves 1* and *2*) and of equivalent amounts of free pyridoxal-P (13 μM, *curves 3* and *4*) in the absence (*curves 1* and *3*) or presence (*curves 2* and *4*) of 4 M NH_4Cl. All solutions were buffered at pH 8.5 with 0.1 M triethanolamine–0.1 mM dithiothreitol–20 mM KCl.

At concentrations of 50 mM and higher the effects of KCl and NH_4Cl are different; ammonia elicits a much greater increase than K^+ in absorbance in the 405- to 420-nm range, so that the sum of the absorbances at 337 and 415 nm increases. At these high concentrations, NH_4^+ (but not K^+) also inhibits degradation of tryptophan by reaction **1**. Complete spectra (not reproduced) show that in 1.0 M KCl, the long wavelength absorption maximum is centered at 410 nm; with 1 M NH_4Cl this peak is considerably intensified and is shifted to 420 nm. The extent of the change at 420 nm is shown in Fig. 7. At pH 8.5, the holoenzyme shows relatively high absorbance at 337 nm and very little absorbance at 420 nm; the converse is true in the presence of excess NH_4Cl (compare *curves 1* and *2*, Fig. 7). Free pyridoxal-P also shows a red shift on addition of NH_4Cl (*curves 3* and *4*, Fig. 7); reduction of the latter reaction mixture with sodium borohydride leads to formation of pyridoxamine 5′-phosphate (unpublished data), showing that the species absorbing maximally at 405 nm is an imine of pyridoxal-P. On this basis, we ascribe the absorption at 420 nm shown by holotryptophanase plus NH_4Cl (*curve 2*, Fig. 7) to formation of an enzyme-bound, coenzyme–NH_3 complex, in which ammonia probably is bound as an imine. Significantly, half-maximal change in absorbance at 420 nm occurs at about a concentration of 300 mM NH_4Cl (pH 8.5), which is very similar to the previously derived K_m value for this substrate for synthesis of tryptophan. Since neither pyruvate nor indole is required for such complex formation, ammonia appears to be the first substrate bound to holotryptophanase in the synthesis of tryptophan from ammonia, pyruvate, and indole. At higher pH values (9.5–10.0), holotryptophanase is partly resolved by 4 M NH_4Cl but not by 4 M KCl. The result again demonstrates specific complex formation with ammonia that results in rupture of the azomethine bond between apotryptophanase and pyridoxal-P, in addition to (at this pH) other bonds that bind coenzyme to apoenzyme.

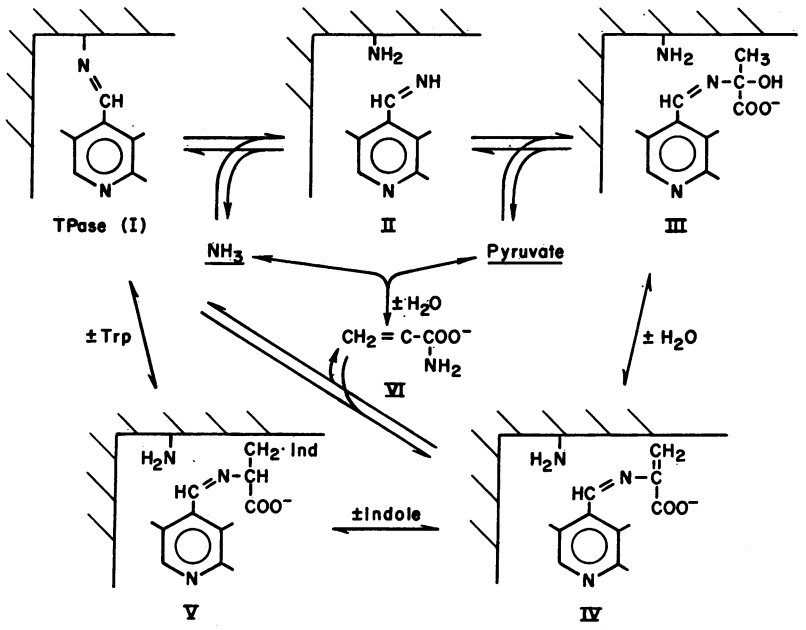

FIG. 8. Possible mechanisms for the reversible degradation of tryptophan by tryptophanase. See text for discussion. The mechanisms are abbreviated: they do not show intermediate aldamines involved in formation or cleavage of enzyme-substrate complexes, or the stepwise transition from V to IV via quinoidal or carbanionic intermediates (15), and they carry no implications concerning the ionic state of the intermediates shown. The abbreviations are: trp, tryptophan; TPase, tryptophanase; Ind, indolyl radical.

DISCUSSION

Previous mechanistic studies of tryptophanase-catalyzed reactions (4, 15) are compatible with either of the two abbreviated mechanisms shown in Fig. 8 for the reversible catalysis of reaction 1. Those studies showed that all amino-acid substrates of tryptophanase gave rise, by elimination of an α-proton and β-elimination of an R group, to a common intermediate, which was visualized as an enzyme-bound α-aminoacrylate (*IV* in Fig. 8). This intermediate could either add indole to form tryptophan (*IV*→*V*→*I* in Fig. 8) or decompose to pyruvate and ammonia, a process visualized as proceeding by transimination within intermediate IV to yield free α-aminoacrylate (*VI*), which then added water with elimination of ammonia to yield pyruvate (Fig. 8). There has been no experimental evidence that favors this route over an equally plausible, but longer, route that involves addition of water to IV to yield III, with formation of pyruvate and ammonia then occurring as a result of transimination within intermediate III or II (Fig. 8). Spectral changes induced in holotryptophanase at high concentrations of ammonia now demonstrate that formation of a complex of unknown structure, but probably closely related to II in Fig. 8, does occur between NH_3 and enzyme-bound pyridoxal-P at concentrations of ammonia similar to those required for tryptophan synthesis. These results, together with the kinetic results that show that pyruvate is the second substrate to combine with enzyme during the synthetic reaction, strongly favor the sequence I→II→III→IV→V→I (Fig. 8) as the catalytic cycle occurring during synthesis of tryptophan, and hence the reverse of this sequence would appear most likely for degradative reactions catalyzed by tryptophanase. The evidence is not yet sufficient, however, to exclude the possibility that free α-aminoacrylate (*VI*) or its tautomer, α-iminopropionate, may also contribute to the degradative or synthetic reactions; the exact structures of the intermediates shown in Fig. 8 are still unknown. Studies of these and other aspects of this reaction are still in progress.

Synthesis of isotopically labeled or unlabeled tryptophan, 5-hydroxytryptophan, and other related amino acids on a preparative scale by reactions described here should be readily possible.

We thank Dr. H. Yamada for discussions of some of his work with β-tyrosinase before its publication. This work was supported in part by research Grants AM-01448 and AI-01575 from the U.S. Public Health Service.

1. Wood, W. A., Gunsalus, I. C. & Umbreit, W. W. (1947) *J. Biol. Chem.* **170**, 313–321.
2. Newton, W. A. & Snell, E. E. (1964) *Proc. Nat. Acad. Sci. USA* **51**, 382–389.
3. Newton, W. A., Morino, Y. & Snell, E. E. (1965) *J. Biol. Chem.* **240**, 1211–1218.
4. Morino, Y. & Snell, E. E. (1967) *J. Biol. Chem.* **242**, 2793–2799.
5. Kumagai, H., Yamada, H., Matsui, H., Ohkishi, H. & Ogata, K. (1970) *J. Biol. Chem.* **245**, 1767–1772.
6. Kumagai, H., Matsui, H., Ohkishi, H., Ogata, K., Yamada, H., Ueno, T. & Fukami, H. (1969) *Biochem. Biophys. Res. Commun.* **34**, 266–270.
7. Ueno, T., Fukami, H., Ohkishi, H., Kumagai, H. & Yamada, H. (1970) *Biochim. Biophys. Acta* **206**, 476–479.
8. Yamada, H., Kumagai, H., Kashima, N., Torii, H., Enei, H. & Okumura, S. (1972) *Biochem. Biophys. Res. Commun.* **46**, 370–374.
9. Kagamiyama H., Wada, H., Matsubara, H. & Snell, E. E. (1972) *J. Biol. Chem.* **247**, 1576–1586.
10. Newton, W. A. & Snell, E. E. (1962) *Proc. Nat. Acad. Sci. USA* **48**, 1431–1439.
11. Udenfriend, S. & Peterson, R. E. (1957) in *Methods in Enzymol.*, eds. Colowick, S. P. & Kaplan, N. O. (Academic Press, New York), Vol. III, pp. 613–614.
12. Cleland, W. W. (1963) *Biochim. Biophys. Acta* **67**, 104–137.
13. Cleland, W. W. (1970) in *The Enzymes*, ed. Boyer, P. (Academic Press, New York and London), 3rd ed., Vol. II, pp. 13–18.
14. Happold, F. C. & Struvenberg, A. (1954) *Biochem. J.* **58**, 379–382.
15. Morino, Y. & Snell, E. E. (1967) *J. Biol. Chem.* **242**, 2800–2809.