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Formation of an Intermediate and Its Rate of Conversion to Pyruvate during the Tryptophanase-Catalyzed Degradation of *S*-*o*-Nitrophenyl-L-cysteine†

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ABSTRACT: The tryptophanase-catalyzed degradation of *S*-*o*-nitrophenyl-L-cysteine (SOPC) to *o*-nitrothiophenol, pyruvate, and ammonia was examined by noting changes in SOPC directly at 330 nm or by coupling the appearance of pyruvate to the oxidation of NADH catalyzed by lactic dehydrogenase. A discrepancy between these two rates suggested the appearance of a solution intermediate which was subsequently trapped by reduction with NaB^3H_4. The resulting [^{3}H]alanine was shown to be racemic by oxidative deamination with D-amino acid oxidase and L-glutamic dehydrogenase. When 200–400 μg of tryptophanase was used with 163–650 μg of lactic dehydrogenase in a coupled reaction mixture, the appearance of pyruvate followed first-order kinetics with the same first-order rate constant, $k = 27.1 \pm 1.08$ min^{-1} at 30 °C. Therefore, the rate-limiting step under these conditions is the nonenzymatic conversion of an intermediate to pyruvate. On the basis of these data and the experiments of Vederas et al. (1978) [Vederas, J. C., Schleicher, E., Tsai, M.-D., & Floss, H. G. (1978) *J. Biol. Chem. 253*, 5350–5354] which showed that the β carbon of pyruvate was stereospecifically protonated during the tryptophanase catalyzed degradation of tryptophan, it is concluded that α-iminopropionate or the carbinolamine of pyruvate and ammoia together with *o*-nitrothiophenol are the immediate products of the tryptophanase-catalyzed degradation of SOPC. The intermediate undergoes a measurable rate of conversion to pyruvate and ammonia.

Tryptophanase catalyzes a variety of reversible α,β-elimination reactions and β-replacement reactions as reviewed by Snell (1975). The reaction involves the enzymatic labilization of the α proton of the Schiff base complex of the amino acid and pyridoxal phosphate with elimination of the β substituent. The resulting aminoacrylate complex can be hydrolyzed or nucleophilic addition can result in β substitution. The reverse reaction appears to involve initial attack by ammonia on the coenzyme followed by addition of pyruvate to form the same aminoacrylate intermediate.

Since the degradation of tryptophan proceeds at a rate comparable to that of the reverse reaction (Watanabe & Snell, 1972), Snell (1975) suggested that the mechanism of degradation was likely the same as the synthesis reaction and, therefore, the hydrolysis of the aminoacrylate intermediate in the degradative pathway was enzyme catalyzed. This suggestion was recently supported by Vederas et al. (1978) when

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they showed that protonation of the β carbon of the aminoacrylate intermediate, to eventually yield pyruvate, was stereospecific.

This paper presents evidence that the tryptophanase-catalyzed degradation of *S-o*-nitrophenyl-L-cysteine (SOPC)[1] yields *o*-nitrothiophenol and an unstable solution intermediate. However, α-aminoacrylate remains enzyme bound long enough for indole to interact in a reverse reaction to give tryptophan. The intermediate is hydrolyzed nonenzymatically and at a measurable rate to pyruvate and ammonia.

Materials and Methods

Holotryptophanase was isolated from *Escherichia coli* as previously described (Suelter et al., 1976a). D-Amino acid oxidase (crystalline suspension from hog kidney), glutamic dehydrogenase (3× crystallized from bovine liver), and lactic dehydrogenase (crystalline from rabbit muscle) were from Sigma Chemical Co., St. Louis, MO. Indole was recrystallized from water before use. Sodium boro[^{3}H]hydride was obtained from ICN Pharmaceuticals, Chemical and Radioisotopes Division, Irvine, CA, with a specific activity of 250 mCi/mmol. *S-o*-Nitrophenyl-L-cysteine was synthesized as described previously (Boyland et al., 1962). All other materials were reagent grade quality.

Tryptophanase was routinely assayed with *S-o*-nitrophenyl-L-cysteine (SOPC) (Suelter et al., 1976b). Unless stated otherwise, a typical 1-mL reaction mixture contained 0.6 mM SOPC, 50 mM KCl, and 50 mM potassium phosphate buffer, pH 8.0. Reactions were initiated by addition of holoenzyme (E_{280} = 0.795 mL mg^{-1} cm^{-1}). Complete reaction progress curves were obtained at 370 nm (E = 1860 M^{-1} cm^{-1}) and analyzed when desired by methods previously described (Yun & Suelter, 1977).

Tryptophanase was also assayed by coupling the appearance of pyruvate to the oxidation of NADH catalyzed by lactic dehydrogenase. Complete reaction progress curves were obtained at 330 nm in 1-mL reaction mixtures containing approximately 0.2 mM SOPC, 0.3 mM NADH, 50 mM KCl, 50 mM potassium phosphate, pH 8.0, and lactic dehydrogenase as indicated. The reaction was initiated by addition of tryptophanase and the decrease in absorbance of NADH at 330 nm was corrected for the absorbance of SOPC and its reaction product, *o*-nitrothiophenol (NTP), measured in a separate reaction mixture. Measured extinction coefficients were as follows: NADH, $E_{330\ nm}$ = 5860 M^{-1} cm^{-1}; SOPC, E_{330nm} = 1410 M^{-1} cm^{-1}; NTP, E_{330nm} = 609 M^{-1} cm^{-1}.

The intermediate was trapped as alanine by reduction with NaB^3H_4 and pyruvate was reduced to lactate. [^{3}H]Alanine was separated from [^{3}H]lactate by thin-layer chromatography using silica gel G as an absorbent. Solvents used were 1-butanol–acetic acid–H_2O (3:1:1) (solvent system 1) and $CHCl_3$–CH_3OH–17% NH_4OH (2:2:1) (solvent system 2). Alanine has an R_f of 0.25 in solvent 1 and 0.65 in solvent 2; lactate has an R_f of 0.5 in solvent 1 and 0.1 in solvent 2. [^{3}H]Alanine and [^{3}H]lactate were also separated by chromatography on Dowex 50-X8. [^{3}H]Lactate eluted with a pyridine (0.02 M) acetic acid buffer, pH 2.5. Two molar pyridine acetate buffer pH 4.85 was used to elute [^{3}H]alanine (Schroeder, 1972). All radioactivity measurements were made with a Packard Tri-Carb scintillation counter.

When indole was added to assay mixtures containing SOPC, its uptake was followed by the method of Kupfer & Atkinson (1964) with the following modifications. The SOPC reaction was stopped by addition of an acidic *p*-dimethylaminobenzaldehyde solution. The 550-nm absorbance was corrected for the contribution of *o*-nitrothiophenol (E_{550nm} = 64.6 M^{-1} cm^{-1}) after noting changes in concentration of SOPC at 370 nm (Suelter et al., 1976b). To correct for the interference in color development by tryptophanase, standard solutions of indole contained the appropriate amount of tryptophanase and absorbance readings were taken after 4 min instead of 20 min.

Stereochemistry of NaB^3H_4 Reduction. [^{3}H]Alanine was separated from reaction mixtures stopped with NaB^3H_4 by column chromatography as described above. After lyophilization to remove the pyridinium acetate elution buffer, the sample was dissolved in 0.02 M sodium pyrophosphate, pH 8.3. The sample was divided into two equal portions, each of which was brought to 1 mL by addition of 0.02 M sodium pyrophosphate, pH 8.3, containing catalase (final concentration, 2.1 μg mL^{-1}). D-Amino acid oxidase was added to one of the two identical portions. To ensure that all of the tritiated D isomers were deaminated, cold D-alanine (1.2 mL of 0.4 mM) was added in 50-μL aliquots at 15-min intervals. The resulting solution along with the control were lyophilized to dryness and counted for remaining radioactivity.

The L isomer of alanine was oxidatively deaminated by L-glutamic dehydrogenase. After [^{3}H]alanine was isolated by chromatographic methods and lyophilized to remove pyridinium acetate, it was dissolved in 2 mL of H_2O. Two equal aliquots of this solution (0.25 mL) were added to separate vessels each containing 0.75 mL of a reaction mixture containing the following reagents: 35 mM glycine, pH 9.0, 6.5 mM L-alanine, 2.5 mm NAD, and [^{3}H]alanine. Glutamic dehydrogenase and lactic dehydrogenase were added and allowed to react for 7 h to ensure completion. After separation of the remaining [^{3}H]alanine from lactate by chromatography and lyophilization to remove pyridinium acetate and dissolution in 2.1 mL of H_2O, 1-mL aliquots were counted to determine remaining radioactivity.

Results

Comparison of Rate of Formation of Pyruvate and o-Nitrothiophenol. The question as to whether or not the enzyme bound α-aminoacrylate was hydrolyzed enzymatically when the degradation of SOPC was catalyzed by tryptophanase was first examined by following the reaction in two ways, that is, by noting the appearance of NTP directly or by following the reduction of pyruvate by coupling the reaction to lactic dehydrogenase (Figure 1). Under the conditions of the assay given in Figure 1, one notes that the rate of appearance of NTP exceeds the rate of formation of pyruvate. The difference becomes larger when the tryptophanase concentration is increased. As indicated later (see Table II), lactic dehydrogenase is not limiting. That the difference in rates of formation of NTP and pyruvate is due to the formation and relatively slow hydrolysis of a free intermediate was demonstrated after trapping the intermediate as alanine with $NaBH_4$ or NaB^3H_4. Similar methods have been used by others (Phillips & Wood, 1965; Hafner & Wellner, 1971; Porter & Bright, 1972) to trap imino acids.

Alanine was detected as a fluorescent spot at an R_f of 0.25 by treatment of thin-layer plates with fluorescamine (Udenfriend et al., 1972) after development with solvent system 1, 1-butanol–acetic acid–H_2O (3:1:1). Both solvent systems 1 and 2 were used to develop plates when the reaction was stopped with NaB^3H_4. Peaks of radioactivity were found to comigrate with alanine and lactate standards when radioscans of thin-layer plates were obtained by counting radioactivity at 1-cm intervals along the plate.

[1] Abbreviations used: SOPC, *S-o*-nitrophenyl-L-cysteine; NTP, *o*-nitrothiophenol.

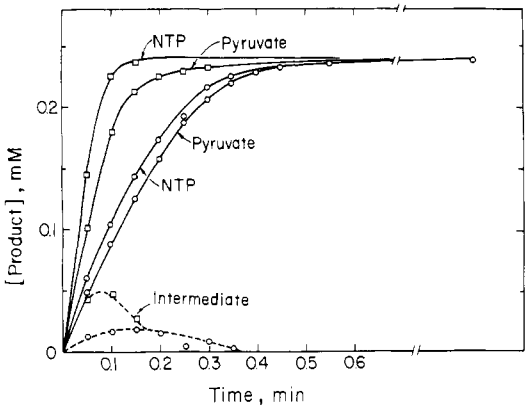


FIGURE 1: Rate of formation of pyruvate and *o*-nitrothiophenol (NTP) during the tryptophanase-catalyzed degradation of SOPC. The 1-mL reaction mixture contained 0.24 mM SOPC, 0.3 mM NADH, 50 mM KCl, 50 mM potassium phosphate, pH 8.0. Rate of *o*-nitrothiophenol was followed by observing the decrease in absorbance at 330 nm (E = 801 M^{-1} cm^{-1}). Pyruvate was measured in a coupled reaction with lactic dehydrogenase (163 μg) by noting the decrease in absorbance at 330 nm. Appropriate corrections were made in the absorbance at 330 nm for the decrease due to the SOPC degradation to determine rate of formation of pyruvate. The reactions were at two concentrations of tryptophanase, (○) 20 μg and (□) 80 μg. The dashed curves in the lower left-hand corner indicate the differences between the rate of formation of NTP and pyruvate at each concentration of tryptophanase and represent the appearance of an intermediate.

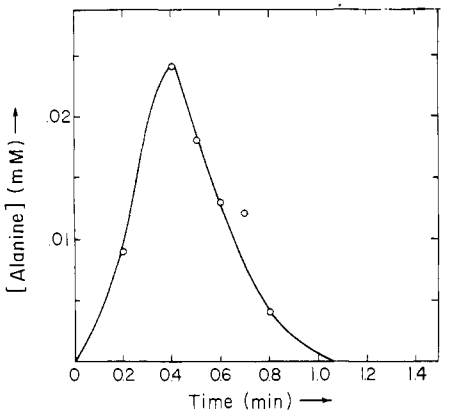


FIGURE 2: Rate of formation of intermediate. Forty microliters of a 1-mL reaction mixture (0.6 mM SOPC, 50 mM KCl, 50 mM potassium phosphate, pH 8.0) stopped at various times with 250 μL of 40 mM NaB^3H_4 was applied onto a silica gel G plate and developed for approximately 4.5 h. Radioscans for each track corresponding to successive points during a reaction were obtained by counting radioactivity at 1-cm intervals along the thin-layer plate. The area under each alanine peak was converted to millimolar concentrations by using the area under the lactate peak at infinite time as a standard for the initial concentration of SOPC.

The intermediate was quantitatively determined after stopping the SOPC reaction at various times by addition of NaB^3H_4. The resulting solutions were chromatographed with solvent system 1 and radioscans of each plate were obtained. The area under each alanine peak was converted to millimolar concentrations by using the area under the lactate peak at infinite time as a standard for the initial concentration of SOPC. As noted in Figure 2, a maximum amount of alanine was detected at 0.4 min, equivalent to 24 nmol, which is considerably in excess of 0.18 nmol of enzyme in the reaction mixture.

Stereochemistry of the NaB^3H_4 Reduction. The [^{3}H]-alanine obtained from a reaction mixture stopped by NaB^3H_4 by column chromatographic methods as described in Materials and Methods was analyzed for stereochemistry by treatment with D-amino acid oxidase and L-glutamic dehydrogenase.

Table I: Stereochemistry of [^{3}H]Alanine Obtained from SOPC Reaction Mixtures Stopped with NaB^3H_4

D-amino acid oxidase[a]		
no treatment	5886 cpm	
after treatment	3694 cpm	
% radioactivity lost	37	
L-glutamic dehydrogenase[a]		
no treatment	25 795 cpm	6345 cpm
after treatment	17 066 cpm	2747 cpm
% radioactivity lost	34	43

[a] [^{3}H]Alanine was oxidatively deaminated as described under Materials and Methods.

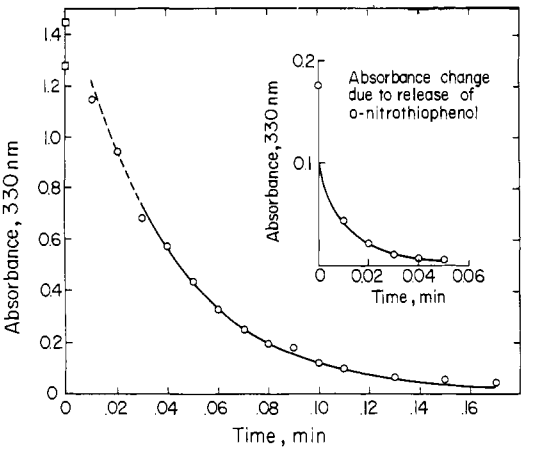


FIGURE 3: Rate of decay of absorbance at 330 nm for a lactic dehydrogenase coupled, tryptophanase-catalyzed degradation of SOPC. The 1-mL reaction mixture contained 50 mM KCl, 50 mM potassium phosphate, pH 8.0, 0.17 mM SOPC, 0.3 mM NADH, and 163 μg of lactic dehydrogenase. The reaction was initiated by addition of 200 μg of tryptophanase and absorbances were recorded with an XKR Sargent-Welch recorder at 20 cm per min. Zero time is the time at which mixing appeared to be complete. The first-order rate constant was calculated with an iterative computer program (Bevington, 1969). The line including the dashed portion is theoretically calculated with a first-order rate constant k_1 = 25.6 min^{-1}. The dashed line is a theoretical extension from the calculated value at 0.03 min. The first three points were not included in the calculation because of the error that arises from the decrease in absorbance of SOPC. The squares (□) in the upper left-hand corner show the extent of the change in absorbance of SOPC at 330 nm.

Table I shows that 37% of the radioactivity associated with the [^{3}H]alanine was lost during treatment with D-amino acid oxidase. When [^{3}H]alanine was oxidatively deaminated by L-glutamic dehydrogenase, 34–43% of the counts were lost. Both experiments show, within experimental error, that [^{3}H]alanine obtained by the NaB^3H_4 reduction is racemic and that the intermediate must be free in solution when reduction occurs.

Rate of Hydrolyses of Intermediate. We reasoned that, if the hydrolysis of the intermediate to pyruvate were relatively slow, the addition of excess tryptophanase should catalyze complete degradation of SOPC in a short time period so that the appearance of pyruvate might be monitored with lactic dehydrogenase. Figure 3 shows the results of such an experiment.

The inset shows the decay of SOPC and growth of NTP observed at 330 nm when lactic dehydrogenase was not added to the reaction mixture. The degradation of SOPC is essentially complete in 0.03 min. However, the appearance of pyruvate as monitored by the lactic dehydrogenase reaction follows a first-order reaction curve and is observed over a longer time period. This result cannot be due to limiting lactic dehydrogenase since the same first-order rate constant, within experimental error, was obtained when the reaction mixture

Table II: Summary of Rates of Hydrolysis of Intermediate

conditions[a]	k_1 (min^{-1})
163 μg of lactic dehydrogenase 200 μg of tryptophanase	25.6 ± 0.37
490 μg of lactic dehydrogenase 200 μg of tryptophanase	26.7 ± 0.28
650 μg of lactic dehydrogenase 200 μg of tryptophanase	27.6 ± 0.28
325 μg of lactic dehydrogenase 400 μg of tryptophanase	28.5 ± 0.49
average	27.1 ± 1.08 SEM

[a] The rate of decay of absorbance was observed at 330 nm for a lactic dehydrogenase coupled, tryptophanase-catalyzed degradation of SOPC. The 1-mL reaction mixture contained 50 mM KCl, 50 mM potassium phosphate, pH 8.0, 0.17 mM SOPC, 0.3 mM NADH. The reaction was initiated by addition of tryptophanase to the reaction mixture containing lactic dehydrogenase as indicated in the table.

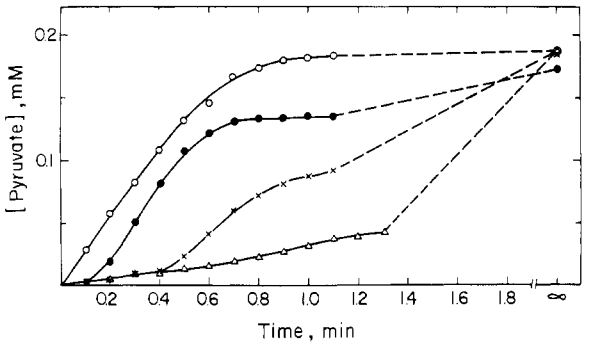


FIGURE 4: Rate of formation of pyruvate in the presence of indole. The 1-mL reaction mixture contained a 0.2 mM SOPC, 50 mM KCl, 50 mM potassium phosphate, pH 8.0, 0.3 mM NADH, and 50 μg of lactic dehydrogenase. The reaction was initiated by addition of tryptophanase. Indole concentrations were (○) no indole; (●) 0.04 mM; (X) 0.1 mM; (△) 0.16 mM. Appropriate calculations were made as described in Materials and Methods to determine the rate of appearance of pyruvate.

contained up to four times as much lactic dehydrogenase or twice as much tryptophanase. The average first-order rate constant of 27.1 min^{-1} gives $t_{1/2}$ = 1.53 s (Table II).

Reverse Reaction with Indole. It was of interest at this stage to determine whether or not α-aminoacrylate remained bound to the enzyme for sufficient time after release of *o*-nitrothiophenol for indole to interact in a reverse reaction to form tryptophan. To test this, differing concentrations of indole were added to SOPC reaction mixtures containing lactic dehydrogenase and NADH so that the yield of pyruvate could be continuously recorded. As noted in Figure 4, the initial rate of pyruvate production is markedly reduced as the concentration of indole is increased. Furthermore, when the rate of pyruvate production levels off at each concentration of indole added, the yield of pyruvate corresponds roughly to the difference between the initial concentration of SOPC and the amount of indole added. After reaction mixtures with different concentrations of indole were allowed to react for 1.2 min and then chromatographed by TLC using solvent system 1, a ninhydrin-positive spot that comigrated with a tryptophan standard was found to increase in intensity as the initial concentration of indole was increased. When indole concentrations were assessed as described in Materials and Methods, they were found to decrease to near zero at the point where the rate of pyruvate production increased (data not shown). However, it is also clear that the yield of pyruvate at infinite time closely approximates the initial concentration of SOPC. The data are clearly consistent with a mechanism in which indole interacts with an enzyme intermediate complex to form tryptophan which, after SOPC is degraded, also undergoes degradation to yield indole, pyruvate, and ammonia.

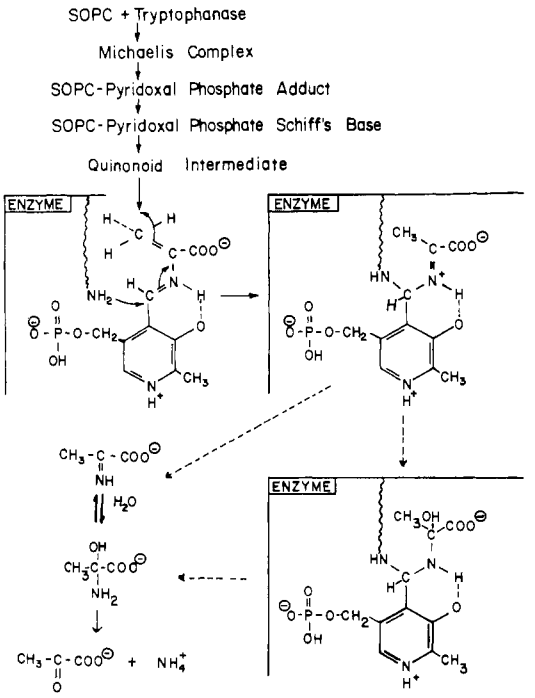
Discussion

Ever since the report of Chargaff & Sprinson (1943), various intermediates have been considered in pyridoxal phosphate dependent enzymes catalyzing α,β-elimination reactions. With respect to tryptophanase, it is now generally accepted that enzyme-bound α-aminoacrylate is formed (Snell, 1975), but there is not agreement on the fate of this intermediate. Previous studies by Phillips & Wood (1965) with threonine dehydrase and by Krongelb et al. (1968) with homoserine dehydratase led Davis & Metzler (1972) to propose that tautomerization of bound α-aminoacrylate is enzyme catalyzed and that α-iminopropionate is released into solution. Snell (1975), after reviewing the evidence available at that time, suggested that α-aminoacrylate is released as such or that the intermediate may be enzymatically hydrated and released as the carbinolamine of pyruvate. Vederas et al. (1978) showed that the β carbon of pyruvate was stereospecifically protonated and as a consequence suggested that pyruvate is eliminated during catalysis.

The data presented in Tables I and II of this paper provide convincing evidence that pyruvate is not released as such during the tryptophanase-catalyzed degradation of SOPC. As indicated in Table II, the production of pyruvate in a lactic dehydrogenase coupled, tryptophanase-catalyzed degradation of SOPC followed first-order kinetics with the same first-order rate constant when 200–400 μg of tryptophanase was used with 163–650 μg of lactic dehydrogenase. Therefore, the rate-limiting step in the reaction under these conditions involves a nonenzymic reaction in which some intermediate is converted to pyruvate. The data of Table I support this conclusion and show that a portion of the intermediate produced during the SOPC reaction can be trapped as alanine by addition of $NaBH_4$. When NaB^3H_4 was used, the resulting alanine was racemic showing that the reduction of the intermediate occurred in solution and not on the enzyme surface.

What is the structure of the intermediate? The experiments of Vederas et al. (1978) show that the β carbon of pyruvate was stereospecifically protonated during the tryptophanase-catalyzed degradation of tryptophan and that α-aminoacrylate, as such, cannot be released during the reaction. Furthermore, on the basis of the considerations clearly delineated by Phillips & Wood (1965) and Szmuszkovics (1963), that is, that enamines are reduced only after tautomeric rearrangement to the imino compound, the intermediate trapped by $NaBH_4$ must be α-iminopropionate. Since α-iminopropionate was trapped as alanine by borohydride reduction, the data might be interpreted as most consistent with the proposal that tautomerization of aminoacrylate is enzyme catalyzed and released as α-iminopropionate. However, as indicated in Scheme I, if the carbinolamine of pyruvate were the first product as originally suggested by Snell (1975), it can disproportionate in two different modes. In the first case, it may decay rapidly to pyruvate and ammonia in which case alanine could not have been trapped. On the other hand, a certain portion may eliminate water and form α-iminopropionate. Because of the lack of rate constants for breakdown of the possible carbinolamine, and the difficulty of present techniques to ascertain these rates, it is not possible to determine whether α-iminopropionate or the carbinolamine of pyruvate and ammonia together with *o*-nitrothiophenol are the immediate products of the tryptophanase-catalyzed degradation of SOPC. It is clear, however, that one of these compounds is the immediate

Scheme I: Schematic Presentation of the Reaction Catalyzed by Tryptophanase[a]



[a] The scheme is meant to portray the flow of the reactions and not the exact details of the process. Most reactions are probably reversible and the exact ionic forms of both coenzyme and substrate are unknown. The dashed line indicates that the breakdown of enzyme-bound α-aminoacrylate may proceed in two alternate pathways.

product of the tryptophanase reaction.

These results do not clarify the kinetic data for the synthesis of tryptophan catalyzed by tryptophanase (Watanabe & Snell, 1972). In the reverse reaction, ammonia reacts with holotryptophanase to form pyridoximine which after addition of pyruvate yields enzyme-bound α-aminoacrylate. Subsequent addition of indole yields tryptophan. However, as suggested by a reviewer, the results reported in this paper and those of Watanabe & Snell (1972) might be explained by assuming parallel pathways of synthesis. At high ammonia concentrations, the pyridoximine pathway might be favored.

One feature of this study, given by the data of Table II and Figure 3, is that the conversion of α-iminopropionate to pyruvate and ammonia proceeds at a relatively slow rate, $t_{1/2}$ = 1.53 s. It is important to realize, therefore, that the apparent rate of α,β-elimination reactions which are follwed by a lactic dehydrogenase coupled assay may under certain conditions be limited by the conversion of α-iminopropionate to pyruvate and not by the enzyme reaction per se.

Acknowledgments

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