Synthesis of L-Tryptophan from Pyruvate, Ammonia and Indole[†]

By Hidetsugu NAKAZAWA, Hitoshi ENEI, Shinji OKUMURA
and Hideaki YAMADA*

Central Research Laboratories, Ajinomoto Co., Inc.,
Kawasaki, Japan

*Research Institute for Food Science,
Kyoto University, Kyoto, Japan

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The tryptophanase activity which synthesizes L-tryptophan from pyruvate, ammonia and indole, was found to be widely distributed in cells of bacteria belonging to *Enterobacteriaceae*, such genera as *Escherichia*, *Kluyvera*, *Enterobacter*, *Erwinia* and *Proteus*. With the cells of *Proteus rettgeri*, equilibrium of the elimination reaction of L-tryptophan in the presence of high concentration of ammonia was studied. It was found that the equilibrium inclines toward the synthetic state.

When 5-hydroxy- and 5-methyl-indole were substituted for indole, 5-hydroxy- and 5-methyl-L-tryptophan, respectively, were synthesized. The synthesis of L-tryptophan was also observed with indole and various amino acids, S-methyl-L-cysteine, S-ethyl-L-cysteine, L-cysteine, 5-fluoro-DL-tryptophan, or oxalacetic acid.

Tryptophanase is an enzyme which catalyzes the stoichiometric conversion of L-tryptophan to pyruvate, ammonia and indole (reaction [1]), and requires pyridoxal phosphate as a cofactor.¹⁾ A homogeneous preparation of the enzyme has been prepared by Newton *et al.*^{2,3)} from *Escherichia coli*.

They reported that the crystalline preparation of enzyme catalyzes a series of α,β -elimination [2] and β -replacement [3] reactions.

In reactions of [2] and [3], R_I may represent -OH, -OCH₃, -SH, -SCH₃ or indolyl radicals and R_{II} represents indolyl radicals.

[†] Bacterial Synthesis of L-Tryptophan and Its Analogues, Part I.

Subsequent to these investigations, we^{4~8)} showed that β -tyrosinase from *Escherichia intermedia* and *Erwinia herbicola* catalyzes the conversion of L-tyrosine to pyruvate, ammonia and phenol (reaction [4]), but not that of L-tryptophan.

It catalyzes a series of α,β -elimination [5] and β -replacement [6] reactions, by a similar reaction mechanism as that of tryptophanase.

In reactions of [5] and [6], R_{III} represents -OH, -SH, or phenolyl radicals, and R_{IV} phenolyl radicals. We⁹⁾ also showed that β -tyrosinase catalyzes the synthesis of L-tyrosine from pyruvate, ammonia and phenol, ap-

parently by the reversal of α,β -elimination reaction.

In appropriate studies, it has recently been proved that the crystalline tryptophanase from *E. coli*¹⁰⁾ and *Proteus rettgeri*¹¹⁾ also catalyzes the synthesis of L-tryptophan by the reversal of α,β -elimination reaction, at rates similar to the forward reaction.

On the basis of these findings, an investigation has been undertaken in our laboratory, for preparation of L-tryptophan or its analogues from pyruvate, ammonia and indole or its analogues, respectively. The present paper describes the distribution of tryptophanase activity in bacteria, and some catalytic properties of the enzyme in *Proteus rettgeri*.

EXPERIMENTAL PROCEDURE

Reagents. 5-Hydroxyindole was purchased from Aldrich Chemical Co. Inc. 5-Methylindole was a gift from Dr. Y. Kanaoka, Hokkaido University, Sapporo, Japan. Pyridoxal phosphate was purchased from Sigma Chemical Company. Other chemicals used in this work were commercial products.

Cultural conditions. The bacterial cells used were preserved in the Central Research Laboratories, Ajinomoto Co. Inc. The cells were grown at 31.5°C for 16 hr on the agar slants of a medium containing 1.0% yeast extract, 1.0% polypeptone, 0.5% KCl and 0.2% L-tryptophan. The cells grown on the slants were used for the study on the distribution of tryptophanase activity in bacteria.

The cells of *Proteus rettgeri* AJ 2770 which were selected for the likely source of tryptophanase were grown in a medium consisting of 0.2% L-tryptophan, 2% soybean protein hydrolyzate, 5% corn steep liquor, 0.17% L-alanine, 0.17% L-isoleucine, 0.17% L-tyrosine, 0.17% L-histidine, 0.1% succinic acid, 0.17% citric acid, 0.01% pyridoxine-HCl, 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.2% KH_2PO_4 in tap water (pH 6.0). The culture was carried out at 31.5°C for 16 hr in 500 ml-shaking flasks containing 60 ml of the medium, under shaking. The cells grown in the medium were harvested and directly used as enzyme for the study on the enzymatic synthesis of L-tryptophan and its analogues.

Assay conditions. Tryptophanase activity was

determined by measuring the amount of L-tryptophan synthesized from pyruvate, ammonia and indole in a reaction mixture containing 20 mg of sodium pyruvate, 20 mg of indole, 50 mg of ammonium acetate, 1 mg of Na_2SO_3 , 3 mg of EDTA, 0.1 mg of pyridoxal phosphate and 4 mg of cells as dried matter, in a total volume of 1.0 ml. After pH was adjusted to 9.0 by 6 N KOH, the mixture was incubated at 37°C for 16 hr.

Analytical methods. (a) L-Tryptophan, 5-hydroxy-L-tryptophan and 5-methyl-L-tryptophan were qualitatively detected by paper chromatography with a solvent system of *n*-propanol-ammonia-water (20: 15: 3). These amino acids were detected under ultraviolet light and by spray with ninhydrin or Ehrlich's reagents.¹²⁾

(b) These amino acids were determined using paper chromatography, followed by the absorbance measurement. A definite amount, 10 μl , of the reaction mixture was applied on a filter paper and developed as described above. The spot of the amino acids was eluted from the paper chromatogram with 5 ml of 0.1 N HCl and the amount of the amino acids was estimated spectrophotometrically by measuring the absorbance at 280 m μ .

(c) Pyruvate was determined by the method of Friedemann and Haugen.¹³⁾

RESULTS AND DISCUSSION

Distribution of tryptophanase activity in bacteria

Tryptophanase activity in 1138 strains of bacteria was assayed by measuring the amount of L-tryptophan synthesized from pyruvate, ammonia and indole. It was found that the enzyme activity was widely distributed in bacteria belonging to *Enterobacteriaceae*, such genera as *Escherichia*, *Kluyvera*, *Enterobacter*, *Erwinia* and *Proteus*, as given in Table I. The amount of L-tryptophan synthesized by the cells of 40 strains was shown in Table II.

Isolation and identification of the reaction product

A reaction mixture contained 30 g of sodium pyruvate, 20 g of indole, 50 g of ammonium acetate, 1 g of Na_2SO_3 , 3 g of EDTA, 0.1 g of pyridoxal phosphate and 4 g of cells *Proteus rettgeri* in a total volume of 1,000 ml. After

TABLE I. DISTRIBUTION OF TRYPTOPHANASE IN BACTERIA^{a)}

Genus	No. of strains tested	No. of strains synthesizing L-tryptophan
<i>Pseudomonas</i>	475	0
<i>Xanthomonas</i>	19	0
<i>Acetobacter</i>	79	0
<i>Aeromonas</i>	7	0
<i>Mycoplana</i>	5	0
<i>Vibrio</i>	4	0
<i>Azotobacter</i>	6	0
<i>Rhizobium</i>	14	0
<i>Agrobacterium</i>	6	0
<i>Alcaligenes</i>	54	0
<i>Achromobacter</i>	61	0
<i>Flavobacterium</i>	89	0
<i>Escherichia</i>	44	8
<i>Kluyvera</i>	5	2
<i>Enterobacter</i>	65	12
<i>Erwinia</i>	131	8
<i>Serratia</i>	44	0
<i>Proteus</i>	14	10
<i>Salmonella</i>	7	0
<i>Clostridium</i>	9	0
Total	1138	40

^{a)} The assay conditions were fully described in the text.

pH was adjusted to 9.0 with 6 N KOH, the mixture was incubated at 37°C for 48 hr. Under these conditions, approx. 23 g of tryptophan was synthesized in the mixture based on paper chromatographic analysis. The tryptophan synthesized was isolated by a procedure given, in Fig. 1, yielding colorless crystals. The crystals were identical with authentic L-tryptophan, in respects of melting point, IR- (Fig. 2) and NMR- (Fig. 3) spectra, optical activity and elementary analysis (Table III).

Apparent equilibrium of elimination reaction by cells of Proteus rettgeri

α,β -Elimination reaction [1] catalyzed by tryptophanase is shown to be reversible.^{10,11)} Then, an apparent equilibrium of the elimina-

TABLE II. SYNTHESIS OF L-TRYPTOPHAN BY CELLS OF BACTERIA BELONGING TO ENTEROBACTERIACEAE^{a)}

Strain tested	L-Tryptophan synthesized (mg/ml)
<i>Escherichia coli</i> ATCC 206	3
" " ATCC 745	2
" " ATCC 3655	1
" " ATCC 7009	1
" " ATCC 10798	5
" " AJ 2594	2
" " AJ 2595	2
" " AJ 2597	3
<i>Kluyvera citrophila</i> AJ 2626	2
" " AJ 2628	2
<i>Enterobacter aerogenes</i> ATCC 8329	9
" " ATCC 8724	11
" <i>liquefaciens</i> AJ 2661	10
" (sp.) AJ 3044	7
" " AJ 3047	7
" " AJ 3049	8
" " AJ 3051	8
" " AJ 3053	1
" " AJ 3054	1
" " AJ 3055	3
" " AJ 3056	1
" " AJ 3080	9
<i>Erwinia herbicola</i> AJ 2973	1
" " ATCC 23822	1
<i>Erwinia carotovora</i> AJ 2753	1
" " AJ 2754	3
" " AJ 2947	4
" " AJ 2950	3
" " AJ 2979	1
" " AJ 2983	1
<i>Proteus rettgeri</i> AJ 2768	10
" " AJ 2769	10
" " AJ 2770	15
" <i>morganii</i> IFO 3848	3
" <i>vulgaris</i> AJ 2764	4
" " AJ 2765	4
" " AJ 2766	4
" " AJ 2767	4
" " AKU 0082	5
" (sp.)	10

^{a)} The assay conditions were fully described in the text.

tion reaction of L-tryptophan by the cells of *Proteus rettgeri* with higher tryptophanase

Reaction mixture (1 liter)

- concentrated *in vacuo*
- adjusted pH to 1.0 with conc. HCl
- centrifuged to separated bacterial cells and residual indole

Supernatant

- concentrated *in vacuo*
- adsorbed on active charcoal
- eluted with 0.1 N HCl
- washed with water
- eluted with 0.3 N NH_4OH

Elute

- concentrated *in vacuo*
- adjusted pH to 5.9 with 6 N HCl

CrystalsRecrystals

FIG. 1. Isolation of the Reaction Product.

TABLE III. PROPERTIES OF L-TRYPTOPHAN

L-Tryptophan	Optical activity	Elemental analyses		
	$[\alpha]_D^{23}(c=0.5 \text{ in } \text{H}_2\text{O})$	C	H	N
Isolated	-31.0	64.9	6.0	14.0
Authentic	-31.0	64.8	5.9	13.7

FIG. 2. Infrared Absorption Spectra of Authentic (A) and Isolated L-Tryptophan (B).

FIG. 3. NMR Spectra of Authentic (A) and Isolated L-Tryptophan (B).

The spectra were recorded with a Varian A-60 NMR spectrometer with DDS as the internal standard.

FIG. 4. Equilibrium of Elimination Reaction of L-Tryptophan by Cells of *Proteus rettgeri*.

The degradation reaction (A) was carried out at 37°C in a reaction mixture containing 25 μ moles of L-tryptophan, 650 μ moles of ammonium acetate, 8 μ moles of Na₂SO₃, 8 μ moles of EDTA, 0.4 μ mole of pyridoxal phosphate and 4.0 mg of cells in a total volume of 1 ml (pH adjusted to 9.0 with 6 N KOH). The synthetic reaction (B) was carried out at 37°C in the same reaction mixture as that of the degradation reaction, except that 25 μ moles of L-tryptophan was replaced by 25 μ moles of sodium pyruvate and 25 μ moles of indole.

activity was examined in the presence of high concentration of ammonia. The result shown in Fig. 4, indicates that the equilibrium inclines toward synthetic state at a level of approximately 80 molar %.

Synthesis of 5-hydroxy-L-tryptophan and 5-methyl-L-tryptophan

When 5-hydroxyindole or 5-methylindole was substituted for indole in the standard reaction mixture, 5-hydroxy-L-tryptophan or 5-methyl-L-tryptophan was synthesized (Table IV) as shown by paper chromatography, and elution time on the automatic amino acid analyzer. In each respect, the product behaved identically with authentic 5-hydroxy-L-tryptophan or 5-methyl-L-tryptophan. How-

TABLE IV. SYNTHESIS OF 5-HYDROXY-L-TRYPTOPHAN AND 5-METHYL-L-TRYPTOPHAN BY CELLS OF *Proteus rettgeri*

The assay mixture containing 20 mg of each substrate as indicated, 30 mg of sodium pyruvate, 50 mg of ammonium acetate, 1 mg of Na₂SO₃, 3 mg of EDTA, 0.1 mg of pyridoxal phosphate and 4 mg of intact cells in a total volume of 1 ml (pH 9.0), was incubated at 37°C for 16 hr.

Substrate	Amino acid synthesized (mg/ml)	
Indole	L-Tryptophan	11
5-Hydroxyindole	5-Hydroxy-L-tryptophan	8
5-Methylindole	5-Methyl-L-tryptophan	5
Sodium sulfide	—	0
2-Mercaptoethanol	—	0
H ₂ O	—	0

TABLE V. SYNTHESIS OF L-TRYPTOPHAN BY CELLS OF *Proteus rettgeri*

The assay mixture containing 30 mg of each substrate as indicated, 20 mg of indole, 50 mg of ammonium acetate, 1 mg of Na₂SO₃, 3 mg of EDTA, 0.1 mg of pyridoxal phosphate and 4 mg of intact cells in a total volume of 1 ml (pH 9.0), was incubated at 37°C for 16 hr.

Substrate	L-Tryptophan synthesized (mg/ml)
Pyruvic acid	14
S-Methyl-L-cysteine	17
S-Ethyl-L-cysteine	15
L-Cysteine	8
L-Cystine	5
L-Serine	5
D-Serine	tr.
D-Tryptophan	0 ^{a)}
5-Hydroxy-DL-tryptophan	1
5-Methyl-DL-tryptophan	2
5-Fluoro-DL-tryptophan	6
Oxalacetic acid	10
DL-Threonine	0

^{a)} Determined by microbiological method with *Leuconostoc mesenteroides*.¹⁶⁾

ever, when indole was replaced by hydrogen sulfite or methyl mercaptan, no synthesis of L-cysteine or S-methyl-L-cysteine was recognized.

Synthesis of L-tryptophan

β -Replacement reactions [3] are shown to be catalyzed by tryptophanase.^{2,3,14)} The synthesis of L-tryptophan from various amino acids and indole via β -replacement reaction was examined with the cells of *Proteus rettgeri*. L-Tryptophan was synthesized from indole and S-methyl-, S-ethyl-L-cysteine, L-cysteine, L-cystine, L-serine, 5-hydroxy-, 5-methyl- or 5-fluoro-DL-tryptophan (Table V). Oxalacetate also reacted, in the presence of high concentration of ammonia, with indole to synthesize L-tryptophan. It can be assumed that oxalacetate may be converted to pyruvate and CO₂¹⁵⁾ under the reaction conditions, then pyruvate formed interacts with ammonia and indole to synthesize L-tryptophan. No synthesis of L-tryptophan was observed with indole and D-form of the amino acids.

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