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Studies on a Species of *Arthrobacter*

II. Tryptophan Metabolism

By

Yôsuke MINO

(*Division of Entomology and Plant Physiology, Obihiro Zootechnical University, Obihiro, Hokkaido, Japan*)

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アースロバクター属の1種に関する研究

II. トリプトファン代謝

美濃羊輔*

Introduction

Two schemes have been reported concerning the complete oxidation of tryptophan (Try) to water and carbondioxide (GREENBERG, 1961 and MEHLER, 1955). One is the pathway through kynurenine, anthranilic acid and catechol and the other through 3-hydroxykynurenine and 3-hydroxyanthranilic acid.

On the other hand, many investigaters have been interested in the plant hormone, indole-3-acetic acid, which is formed from Try (RIDDLE and MAZELIS, 1965). MINO (1958 a and b) has shown that a species of *Arthrobacter*, which was isolated from the air, can utilize indole-3-acetic acid and L-tryptophan as its sole source of carbon and nitrogen. This paper describes a new scheme of complete oxidation of Try through IAA by this bacterium.

Materials and Methods

Organism and its culture

A species of *Arthrobacter* was used throughout this study. A loopful of bacterium from slant culture was inoculated into 200 m*l* of phosphate buffer (M/15, pH 6.8) containing 1% polypeptone, followed by culturing at 30°C for 16 hr without shaking. Bacterial culture for the investigation of IAA metabolism was carried out as described elesewhere (MINO, 1968 a).

Determination of bacterial cell concentration, oxygen uptake by the bacterium and Salkowsky reagent positive compounds

Bacterial cell concentration was determined by the measurement of optical density at 500 mμ with a spectrophotometer (Hitachi, Model 101). Oxygen uptake

* 帯広畜産大学昆虫学・植物生理学教室

was measured with a Warburg manometer, in the usual way, at 30°C. The reaction mixture consisted of 2 m*l* of bacterial suspension in the main chamber and 0.2 m*l* of substrate in the side arm, unless otherwise stated. For the determination of Salkowsky reagent positive compounds, 3 m*l* of 36% $HClO_4$ containing 0.01 M $FeCl_3$ were added to 1 m*l* of the reaction mixture from which the bacterial cells were previously filtered off. The color, developed at 30°C for 25 min, was measured at 530 mμ with the spectrophotometer.

Solvent systems and spray reagents for paper chromatography

Solvent A composed of ethanol-water (7 : 3), B of benzene-*n*-butanol-methanol-water (2 : 2 : 4 : 1), C of *n*-butanol-acetic acid-water (4 : 1 : 5), D of water-acetic acid (98 : 2), E of pyridine-*n*-butanol-water saturated with NaCl (1 : 1 : 2), F of *iso*-propanol-water (5 : 95), G of *iso*-propanol-ammonia (28%)-water (8 : 1 : 1), H of water and I of benzene-acetic acid-water (8 : 1 : 1).

Fe ; 2% $FeCl_3 \cdot 6H_2O$ in water. NaOH : 4% NaOH in water. DB : 100 mg of stabilized Zn salt of diazotized-4-benzoyl-amino-2, 5-dimethoxyaniline in a mixture of 5 m*l* of dioxane and 10 m*l* of water. NB : 60 mg of *p*-nitrobenzene-diazonium fluoroborate in a mixture of 5 m*l* of dioxane and 10 m*l* of water. B–HCl : 0.1% benzidine in N HCl. DNPH : 500 mg of 2, 4-dinitro-phenylhydrazine in 100 m*l* of N HCl. $NaNO_2$: 0.1% $NaNO_2$ in N HCl. DC : 50 mg of 2, 6-dibromoquinone-4-chloroimide in a mixture of 12 m*l* of dioxane and 3 m*l* of dry acetone. AP : 0.5% aqueous solution of azobenzene-phenylhydrazine sulfonic acid upon succesive spraying with N HCl. BH : 150 mg of hydroxylamine·HCl in 14 m*l* of bromophenol blue solution and 1 m*l* of water. The pH of the solution was adjusted to 7.2 with a few drops of 2 N NaOH. BPB : 300 mg of bromophenol blue in 500 m*l* of ethanol and 0.25 m*l* of 30% sodium hydroxide. EH : 0.5% *p*-dimethylaminobenzaldehyde in N HCl. NH : 2% ninhydrin in *n*-butanol saturated with water. PO : polyphenoloxidase solution. Paper chromatography was carried out by an ascending technique at room temperature using Tôyo filter paper No. 51 A.

Preparation of polyphenoloxidase from potato tubers

One hundred grams of potato tubers were minced and ground with a mixer in phosphate buffer (10^{-2} M, pH 7.0). The pulp was pressed out through a nylon cloth and then centrifuged at 3,000 *g* for 15 min. The extract gave about 100 m*l* of brown fluid, which was then left in a cool place for 4 hr. Acetone (−20°C) was added to the fluid up to 30% by volume, followed by centrifugation at 2,500 *g* for 10 min. The precipitate was discarded. Cold acetone was further added to the supernatant liquid to 60% by volume to collect the precipitate. The colored precipitate was dissolved in deionized water to dialyse against tap water overnight. To the dialysed solution was added solid ammonium sulfate up to 0.3 saturation degree, followed by centrifugation at 3,000 *g* for 15 min to remove the precipitate. More solid ammonium sulfate was then added to the supernatant liquid up to

0.7 saturation degree. The precipitate was suspended in 10 m*l* of deionized water and dialysed against tap water overnight. The solution was used as crude polyphenoloxidase.

Spectrophotometry

Ultraviolet absorption spectrum was obtained in a quartz cuvette of 1-cm light path with a Hitachi recording spectrophotometer.

IAA was purchased from Tôkyo Kasai Co. Ltd. and L-tryptophan from Wako Pure Chemical Industries, Ltd. Indole-3-acetamide was synthesized by the method of Crosby et al. (1960).

Results

IAA oxidation by Try induced and non-induced cells

Bacterial cells were harvested from 400 m*l* of polypeptone medium and washed with 0.9% NaCl solution by centrifugation. The cells obtained was Try-non-induced. The Try-non-induced cells were resuspended in 100 m*l* of phosphate buffer (M/15, pH 6.8) containing 100 mg of Try and shaken at 30°C for 3 hr. Try-induced cells collected were washed with 0.9% NaCl solution. The cells were diluted with phosphate buffer (M/15, pH 7.2) to the concentration of 0.4 OD_{500}. Oxygen uptake was measured manometrically. Manometers were read at intervals of 10 min.

As shown in Fig. 1, IAA was oxidized immediately by Try-induced cells, while IAA was oxidized after some induction period by non-induced cells. This suggests that Try is metabolized through IAA. The data are not described here, but IAA was also oxidized by Try-induced cells in the presence of 10^{-2} M chloramphenicol, which inhibits protein synthesis.

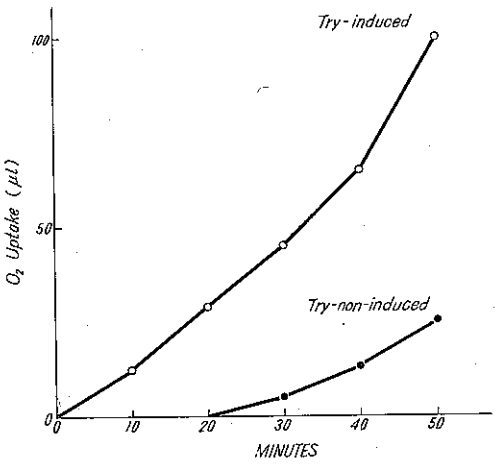

Fig. 1. IAA oxidation by Try-induced and non-induced cells

Variation of Salkowsky reagent positive products in the course of Try degradation

The bacterial cells where collected from 400 m*l* of polypeptone medium and washed with 0.9% NaCl solution. The cells collected were suspended in 100 m*l* of phosphate buffer (M/15, pH 7.2) containing 100 mg of Try and shaken at 30°C. This reaction mixture was pipetted out at intervals of 1 hr. To 1 m*l* of the mixture, from which the cells were previously centrifuged off, were added 3 m*l* of Salkowsky reagent.

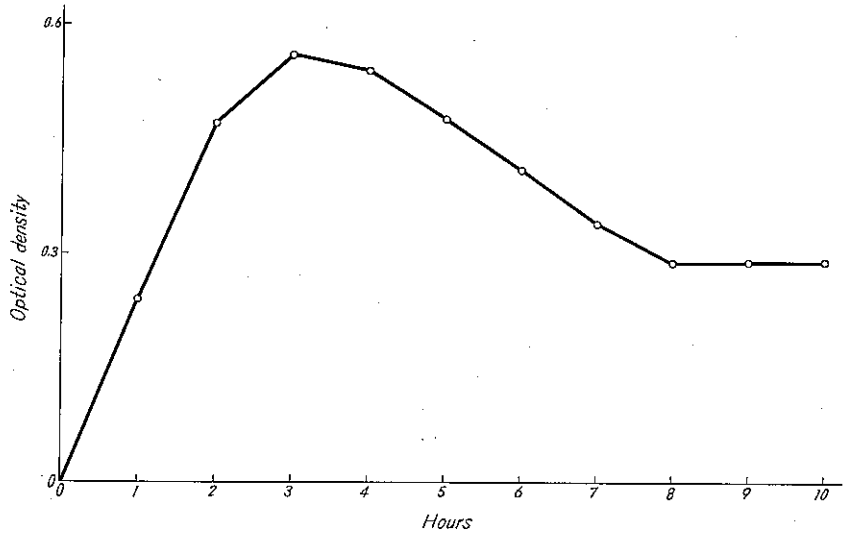

Fig. 2. Variation of Salkowsky reagent positive products in the course of Try degradation

Fig. 2 shows the variation of Salkowsky reagent positive compounds in the reaction mixture. The amount of Salkowsky positive compounds in the reaction mixture was rapidly increased during the first 3 hr, after which it gradually decreased until the constant level was attained. This result indicates that Salkowsky reagent positive compounds were produced from Try and metabolized further. After 8 hr reaction, the amount of these compounds was constant, suggesting that the enzyme system to decompose intermediary metabolites was inactivated as demonstrated in the case of IAA oxidase in this *Arthrobacter* (MINO, 1968c).

Identification of IAA by paper chromatography and ultraviolet spectrophotometry

The bacterial cells were collected from 400 m*l* of polypeptone medium, followed by washing. The cells collected were suspended in 100 m*l* of phosphate buffer (M/15, pH 7.2) containing 100 mg of Try and shaken at 30°C for 6 hr. Then the cells were centrifuged off. The supernatant solution was adjusted to pH 8.0 with N NaOH. To this solution were added 100 m*l* of ethylacetate to extract ethylacetate soluble compounds. Then the water layer was adjusted to pH 3.0 with N HCl and ether soluble compounds were extracted with an equal amount of ether. Ethylacetate and ether extract were condensed to 2 m*l* under a reduced pressure, subjected to paper chromatography. Three spots (1, 2 and 3) from ether extract and one spot from ethylacetate were detected on paper chromatogram developed by solvent C. As presented in Table 1, spot 2 obtained from ether extract seemed to be IAA, since R_f values of authentic IAA were closely identical with those of spot 2. Both IAA and spot 2 showed similarly reaction with Ehrlich reagent.

Spot 2 was eluted with methanol from paper chromatogram to determine

Table 1. R_f values of authentic IAA and spot 2

Solvent system	IAA	Spot 2
A	0.88	0.88
C	0.83	0.83
G	0.40	0.42
I	0.68	0.68

IAA was detected with ultraviolet light.

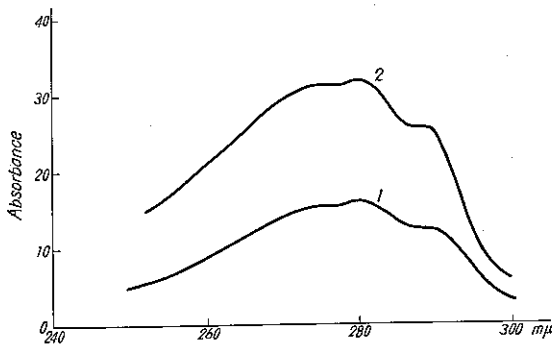

Fig. 3. Ultraviolet spectra of authentic IAA and the eluate from spot 2

the ultraviolet spectrum. In Fig. 3 are shown the ultraviolet spectra of an eluate from spot 2 and authentic IAA. The spectrum of authentic IAA was well comparable with that of the eluate, supporting again that spot 2 was IAA.

Ultraviolet spectra of IAA oxidation products

The cells collected from polypeptone medium were suspended in 100 m*l* of phosphate buffer (M/15, pH 7.0) containing 20 mg of IAA to start the reaction

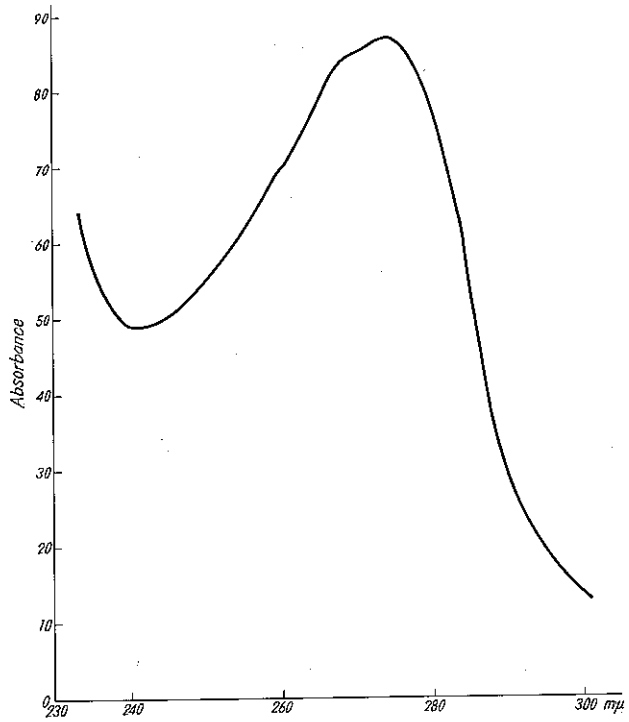

Fig. 4. Ultraviolet spectrum of IAA oxidation products, as observed in the reaction mixture

Table 2. R_f values of catechol and an oxidation product of IAA

Solvent system	Catechol	Oxidation product	Solvent system	Catechol	Oxidation product
A	0.94	0.94	D	0.73	0.72
B	0.90	0.90	E	0.87	0.89
C	0.77	0.77	F	0.82	0.82

Table 3. Color reactions of catechol and an oxidation product

Reagent	Catechol	Oxidation product	Reagent	Catechol	Oxidation product
Fe	dark gray	dark gray	DC	pink	pink
NaOH	yellow	yellow	AP	—	—
DB	gray	gray	BH	—	—
B-HCl	—	—	BPB	—	—
NB	orange	orange	EH	pink	pink
DNPH	—	—	NH	—	—
$NaNO_2$	yellow	yellow	PO	yellow	yellow

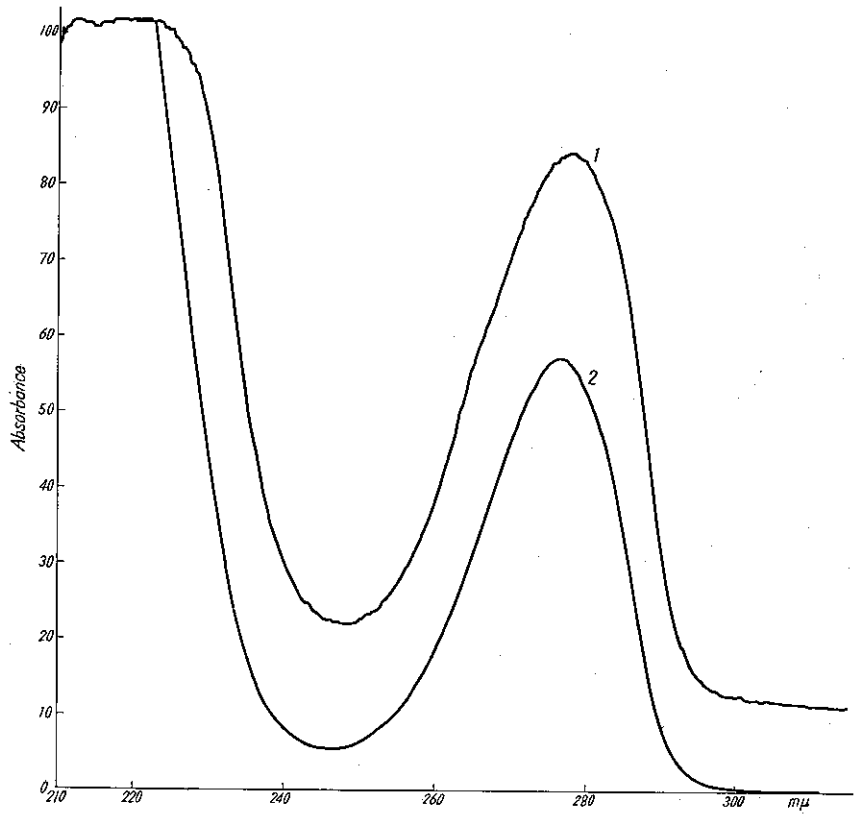

Fig. 5. Ultraviolet spectra of authentic catechol and an eluate

with continuous shaking at 30°C. Immediately after IAA disappeared from the reaction mixture, cells were centrifuged off. This supernatant fluid was subjected to the ultraviolet spectrophotometry. It is seen in Fig. 4 that a compound having a strong peak at 275 mμ was produced from IAA in the reaction mixture.

Identification of a decomposition product from IAA

To the reaction mixture was added an equal volume of ether to extract ether soluble substances. The ether solution was condensed to 5 m*l* under a reduced pressure to be subjected to paper chromatography. In Table 2 are shown R_f values of an oxidation product and authentic catechol. In Table 3 are shown the color reactions of an oxidation product and authentic catechol, developed by various spraying reagents. It is seen in Tables 2 and 3 that R_f values and color reactions are well comparable with those of authentic catechol. This compound was eluted with water from paper chromatogram to determine ultraviolet spectrum. The result in Fig. 5 supports again that an oxidation product of IAA is catechol.

Examination of intermediary metabolites by sequential induction method

Compounds tested were indole-3-acetamide, tryptamine, skatole, anthranilic acid and salicylic acid. Oxidation of these compounds by Try-induced cells was examined manometrically. All these compounds were not oxidized. This seems to reflect that these compounds were not intermediary metabolites of IAA oxidation. Catechol was further oxidized. So there seems to be another complete oxidation system of Try in this *Arthrobacter*.

Discussion

A number of pathways of Try metabolism have been established in a wide field including animals, plants and microorganisms (MEHLER, 1955; PRICE, 1959 and GREENBERG, 1961). KNOX and MEHLER (1950) reported the conversion of Try to kynurenine in liver. This pathway has been also known in microorganisms. On the other hand, the conversion of Try to tryptamine, indole-3-pyruvate (MARTIN and DURHAM, 1966), indole-3-acetamide (RIDDLE and MAZELIS, 1965; KOSUGE *et al.*, 1966 and HUTZINGER and KOSUGE, 1967) or 5-hydroxytryptophan has been roported. However, Try metabolism through kynurenine is the only pathway of its oxidation to completion reported earlier. In this study, Try was shown to be oxidized completely through IAA and catechol, suggesting that a different pathway of complete oxidation of Try is present in this *Arthrobacter*. Though the intermediary metabolites of Try degradation to IAA are not yet clear, Try seems to be metabolized through indole-3-pyruvate, since indole-3-acetamide and tryptamine were not detected in its degradation products. This may be supproted again by the fact that Try-induced cells were not capable of metabolizing these two compounds.

PROCTOR (1958) proposed a scheme of IAA oxidation through skatole, 3-

hydroxyindole and 2, 3-dihydroxindole using a species of *Pseudomonas*. In this connection, however, all these compounds were not detected in the decomposition products of IAA, indicating that IAA seems to be metabolized through a different path from the scheme proposed by him. This may be supported again by no participation of Try-induced cells in the metabolism of these compounds.

In view of the report by RIVIÈRE (1964) concerning the involvement of an *Arthrobacter* in the formation and destruction of IAA in the rhizosphere, more detailed investigations are required to clarify a series of intermediary metabolites of Try degradation to catechol in this bacterium.

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Summary

The degradation pathway of tryptophan was examined using a species of *Arthrobacter*.

Indole-3-acetic acid was identified as an intermediary metabolite. This was metabolized further through catechol. Indole-3-acetamide, skatole and tryptamine might not be regarded as intermediates. This scheme seems to be different from the complete oxidation pathway of tryptophan through kynurenine reported earlier.

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摘　　要

一種のアースロバクターによるトリプトファンの代謝が調べられた。中間代謝産物として，インドール酢酸とカテコールが同定された。トリプトファンからインドール酢酸への転換はインドールピルビン酸を中介して行なわれるものと考えられる。今までに報告されているトリプトファンの完全酸化経路はキヌレニンを経由するもののみであるが，本菌には新しいトリプトファンの完全酸化系が存在しているものと思われる。