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ENZYMATIC CONVERSION OF ANTHRANILIC ACID TO CATECHOL BY CHLOROPLAST FROM THE LEAVES OF *TECOMA STANS*

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Abstract—An enzyme system which converts anthranilic acid to catechol was detected in the leaves of *Tecoma stans*, and its properties studied. The system is present exclusively in the chloroplast fraction of the leaves. The optimum pH of the reaction is 5·2 and maximum activity was obtained with citrate–phosphate buffer. There was good stoichiometry between the amounts of anthranilic acid disappeared and the amounts of catechol and ammonia formed. The enzyme system showed an absolute requirement for oxygen and evidence was obtained for the probable participation of NADPH and FAD in the hydroxylation step. The optimum concentration of anthranilic acid was 10^{-4} M; at higher concentrations the reaction was inhibited to a considerable extent. Cyanide, pyrophosphate, and EDTA also caused inhibition indicating a requirement for metal ions.

INTRODUCTION

IN SPITE of the widespread occurrence of phenolic compounds in plants[1] and their importance in metabolism, very little is known about the enzymes involved in the synthesis of these compounds. Stanier and co-workers[2,3] by using the technique of "sequential induction" (simultaneous adaptation) observed that in certain strains of *Pseudomonas fluorescens* adapted to tryptophan, catechol is formed from anthranilic acid. There is no well-documented evidence for the existence of this pathway in other organisms. As a part of a study of the biosynthesis of polyphenols and also with a view to elucidating the mechanism of tryptophan degradation in the plant kingdom, the possibility that this reaction might occur in plants has been investigated.

Tryptophan and other related compounds, such as anthranilic acid, were therefore tested as possible precursors of catechol, and a number of plant tissues were examined. It was found that washed chloroplast preparations from leaves of *Tecoma stans* readily converted anthranilic acid to catechol. The general properties of the chloroplast enzyme system catalysing the overall reaction are presented in this communication. The plant enzyme system bears a close resemblance to that from *Pseudomonas* reported by Stanier,[2,3] in that it is highly labile and exhibits an obligatory requirement for oxygen. There was practically no anthranilic acid degradation when the reaction was carried out under anaerobic conditions. As the enzyme was completely inactivated by shaking for a short time at 30° in Warburg vessels, oxygen uptake studies could not be carried out. In all probability the overall reaction is brought about by a multi-enzyme system, the chemical nature of the intermediates being not yet

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[3] R. Y. STANIER and O. HAYAISHI, *J. Bact.* **62**, 690 (1951).

known. It has been suggested that salicylic acid might be an intermediate in the formation of catechol from benzoic acid in *P. convexa.* var. *hippuricum*,[4] although, simultaneous adaptation studies by Stanier and co-workers[5,6] with related organisms would seem to contradict this view. In the *Tecoma* chloroplast system, however, salicylic acid was completely inert, and this would tend to rule out its role as an intermediate. One of the important points of difference between the present system and that from *Pseudomonas* is that tryptophan does not seem to

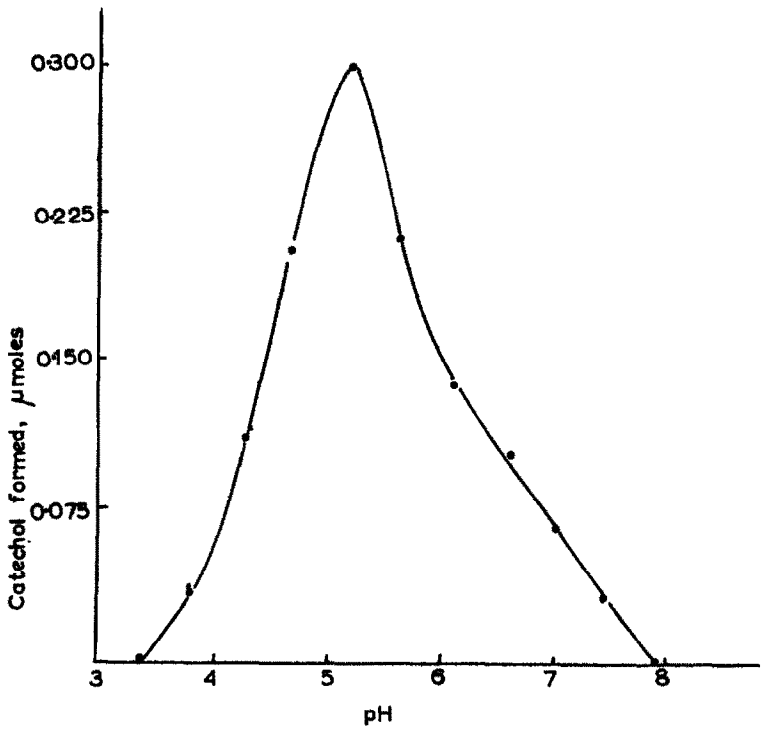

FIG. 1. EFFECT OF pH ON THE CONVERSION OF ANTHRANILIC ACID TO CATECHOL BY THE CHLOROPLAST PREPARATION.

The reaction mixture consisted of 0·5 ml citrate-phosphate buffer at various pH's indicated, 0·4 μmoles of anthranilic acid and 1 ml enzyme preparation in a total volume of 2 ml.

be a precursor of anthranilic acid. Whereas in *Pseudomonas*, the anthranilic acid oxidizing system was induced by adapting the organism to tryptophan, in *Tecoma* leaves it would appear that the enzyme system is constitutive in nature and may have an important function in the normal metabolism of the plant.

The Effect of pH on the Enzyme Reaction

The effect of pH's between 3·4 and 8·0 on the anthranilic acid oxidation by washed chloroplasts from the leaves of *Tecoma stans* is shown in Fig. 1. A single peak at 5·2 was obtained when citrate (0·1 M) phosphate (0·2 M) buffer was used. On the alkaline side the activity

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drops rapidly and at pH 7·4 it is less than 10 per cent of the maximum. The optimum pH 5·2 for the chloroplast enzyme system is markedly different from that reported for *Pseudomonas* enzyme by Stanier, viz. pH 7·5. The effect of different buffers at the optimum pH 5·2 is shown in Table 1 and it is noteworthy that the activity was about 30 per cent higher in the presence of citrate–phosphate buffer than when other buffers were used. Citrate–phosphate buffer was routinely used in subsequent experiments.

TABLE 1. EFFECT OF DIFFERENT BUFFERS AT OPTIMUM pH (pH 5·2) ON ANTHRANILIC ACID OXIDATION

Buffer*	Catechol formed (μmole)
Citrate–phosphate buffer 0·1/0·2 M	0·272
Succinate–sodium hydroxide	0·191
Phthalate–sodium hydroxide	0·163
Acetate–sodium acetate	0·180
Maleate–sodium hydroxide	0·194

* All the buffers used in this experiment were prepared according to G. Gomori, *Methods in Enzymology*, Vol. I, pp. 138–46, Academic Press Inc., New York, 1955.

The reaction vessels contained: 0·5 ml buffer, 0·4 μM anthranilic acid, 1·0 ml enzyme preparation in a total volume of 2·0 ml. The reaction was run at 30° for 15 min and the catechol estimated as described.

Intracellular Distribution of the Enzyme

The leaf homogenates of *Tecoma stans* were subjected to differential centrifugation, as described under Experimental and each of the fractions (chloroplast, mitochondria, supernatant) was assayed for anthranilic acid oxidase activity. All the measurable activity was

TABLE 2. STOICHIOMETRY OF THE REACTION

Time (min)	Anthranilic acid disappeared (μmole)	Ammonia formed (μmole)	Catechol formed (μmole)
10	0·218	0·220	0·230
15	0·262	0·259	0·264
20	0·262	0·260	0·261

The standard system was used (see Table 1 and Experimental) and the anthranilic acid lost and ammonia formed estimated as described in the Experimental.

concentrated in the chloroplast fraction. The mitochondrial and supernatant fractions contained a powerful phenolase so only anthranilic acid disappearance was measured in their case.

Stoichiometry

The stoichiometry of the reaction was next investigated. From the results given in Table 2, it will be clear that anthranilic acid was consumed with concomitant formation of equimolar amounts of catechol and ammonia. Essentially no enzymatic degradation of catechol in the presence of *Tecoma* chloroplasts was observed. As the enzyme was completely inactivated within twenty minutes, experiments could not be continued beyond this period.

Co-factor Requirements

The effect of probable cofactors like flavin adenine dinucleotide (FAD), ascorbic acid, and reduced nicotinamine adenine dinucleotide phosphate (NADPH) was studied. Of the cofactors studied, NADPH alone showed stimulation of catechol formation. The results are given in Table 3. Stimulation of enzyme activity by NADPH is also of interest and has been

TABLE 3. CO-FACTOR REQUIREMENTS

pH of the reaction	Addition*	Catechol formed (μmole)
5·2	Control	0·264
	FAD	0·264
	Ascorbic acid	0·263
6·2	Control	0·072
	NADP†	0·272

* FAD and ascorbic acid (2 μM) were added to normal reaction mixture.

† NADPH was generated *in situ*; the reaction mixture contained, in addition to the usual comconents 0·2 ml NADP (0·1 μmole), 0·1 ml glucose-6-phosphate (2·5 μmoles) and 0·04 ml of glucose-6-phosphate dehydrogenase. The pH of the reaction was adjusted to 6·2 to facilitate the enzymatic reduction of NADP.

The reaction was carried out as described under Table 1.

observed in various hydroxylation reaction.[7–10] For instance NADPH has been reported to activate the non-specific hydroxylation catalysed by liver microsomes.[7] Other examples of NADPH requiring systems are the enzymatic hydroxylations of phenylalanine to tyrosine[8] and the conversion of kynurenine to 3-hydroxy kynurenine by rat liver mitochondria.[9] Saito *et al.*[11] were able to solubilize kynurenine hydroxylase and also achieved a partial purification of the enzyme, so that the reaction occurred with stoichiometric utilization of NADPH and oxygen. FAD did not exert any activating effect on the anthranilic acid oxidase system. However, the participation of Flavin co-enzymes in the reaction is indicated by the inhibition caused by atabrine.

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Effect of Substrate Concentration

The effect of anthranilic acid concentration on the rate of the reaction showed that under the conditions used the optimum substrate concentration was found to be 10^{-4} M. There was a marked drop in activity at higher substrate concentrations and no activity could be detected at 5×10^{-3} M.

Effect of Metal Ions

The effect of different metal ions on the reaction was investigated, and of those tested Zn^{2+}, Mg^{2+} and Co^{2+} ions were found to give 68, 80, and 90 per cent inhibition respectively at 5×10^{-4} M, Hg^{2+}, Mn^{2+} and Fe^{2+} had no effect at this concentration.

Effect of Inhibitors

The enzymatic formation of catechol from anthranilic acid with the standard system was completely inhibited by low concentration of *p*-chloromercuric benzoate (5×10^{-4} M) suggesting the importance of intact sulfhydryl groups. The enzyme was also completely inhibited by equivalent concentrations of atabrine, hydroxylamine, cyanide, and ethylenediaminetetracetate (EDTA), and almost completely by sulphanilamide and pyrophosphate. *p*-Amino benzoic acid gave 43 per cent inhibition at this concentration. The involvement of metal ions could be inferred from the marked inhibition caused by EDTA, pyrophosphate and cyanide. However, the complete absence of inhibition by azide, 8-hydroxy quinoline and O-phenanthroline is somewhat puzzling. It is possible that permeability factors might be responsible for this anomalous result. The fact that the enzyme system is also not inhibited by diethyldithiocarbamate would indicate that copper enzymes are not involved, and would tend to rule out the possibility of participation of phenolase complex of enzymes in this aromatic hydroxylation reaction. The striking inhibition of the oxidation of anthranilic acid by structural analogues, viz. *p*-aminobenzoic acid and sulfanilamide might in all probability be due to competition for the active centres of the enzyme molecule.

EXPERIMENTAL

Isolation of Chloroplasts

All the operations described were carried out in the cold (0–5°). Fresh, mature leaves (12 g) were taken, the blades detached from midribs and ground with acid-washed sand and 0·35 M saline (20 ml). The ground mass was squeezed through a double layer of cheese-cloth and centrifuged for 1 min at 50*g* to remove whole cells and cell debris. The supernatant was then centrifuged for 5 min at 1000*g*.

The residue was resuspended in half the original volume of 0·35 M saline and centrifuged for 1 min at 50*g* to remove clumped chloroplasts. Microscopic examination of the supernatant suspension showed it to consist mainly of whole chloroplast. This fraction was washed three times with 20 ml of saline and finally the chloroplasts were taken in 10 ml 0·35 M saline.

The supernatant obtained after centrifugation for 5 min at 1000*g* (S_1) was centrifuged at 20,000*g* for 20 min to remove the mitochondrial fraction. The supernatant (S_2) from above contained microsomes and was used as the "soluble fraction". The residual pellet which contained mitochondria was resuspended in 10 ml 0·35 M saline medium and designated as the mitochondrial fraction.

Assay Method for the Anthranilic Acid Oxidase System

The enzyme assays were conducted in test tubes containing 0·5 ml of citrate (0·1 M) phosphate (0·2 M) buffer pH 5·2, 0·4 μmole of anthranilic acid and 1 ml enzyme preparation in a total volume of 2 ml. Controls without enzyme and without substrate were run. The tubes were incubated for 15 min at 30°.

For estimation of anthranilic acid the reaction was stopped by the addition of 0·5 ml 30% trichloroacetic acid. The volume was adjusted to 3 ml, the mixture filtered and 0·5 ml of the filtrate was taken for anthranilic acid estimation by the colorimetric method of Venkataraman *et al.*[12]

The catechol formed in the reaction mixture was estimated by the colorimetric method developed by the authors.[13] After incubation, the reaction was stopped by the addition of 0·5 ml formaldehyde (37–41%) in order to destroy the residual anthranilic acid; 5 min later, 0·5 ml 30% trichloroacetic acid was added. The mixture was filtered and to a 1 ml aliquot of the filtrate was added 1 ml of sodium tungstate (10% w/v), 0·5 ml 0·5 N HCl and 1 ml sodium nitrite in that order. After 5 min 2 ml of 0·5 N NaOH was added and red colour produced measured after 15 min.

The ammonia formed from anthranilic acid as a result of enzymatic oxidation was determined by aerating the reaction mixture in the presence of 10% sodium hydroxide and trapping the ammonia formed in Nessler's reagent.[14]

Isolation of the Product of Enzymatic Oxidation of Anthranilic Acid

To 25 ml solution containing 10 mg of anthranilic acid, 25 ml citrate phosphate buffer (pH 5·2), and 50 ml chloroplast suspension obtained from the leaves of *Tecoma stans* were added. After incubating for 15 min, the pH was adjusted to 2·0 with dilute H_2SO_4 and the precipitate formed was removed by centrifugation. The supernatant was cooled and extracted twice with equal volumes of peroxide free ether. The ether extracts were pooled and washed with 10% Na_2CO_3 solution until they no longer fluoresced. The ether extract was concentrated to dryness *in vacuo*. The residue was taken up in 10 ml water. To this solution was added 3 ml 20% lead acetate solution. The white precipitate formed was removed by centrifugation, washed several times with water and finally suspended in 10 ml water. The lead complex was decomposed with H_2S and the mixture filtered and the filtrate extracted twice with equal volumes of peroxide free ether. The ether layer was evaporated to dryness *in vacuo*. The residue thus obtained was dissolved in minimum amount of hot benzene. On cooling, crystals were obtained, which had a melting point of 103°, undepressed after mixing with the authentic sample of pyrocatechol. Paper chromatography in three different solvent systems, (Toluene:acetic acid:water, 4:1:5; Ethanol:ammonium hydroxide, (sp. gr. 0·880):water, 18:1:1; Chloroform:methanol:formic acid:water, 10:1:0·04:0·96) gave same R_f value for pyrocatechol and isolated product.

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