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ANTHRANILIC ACID OXIDASE SYSTEM OF *TECOMA STANS*—III.

STUDIES ON THE CONVERSION OF *O*-AMINOPHENOL TO CATECHOL

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Abstract—The terminal step in the oxidation of anthranilic acid to catechol by anthranilic acid oxidase system from *Tecoma stans*, which converts *o*-aminophenol to catechol has been studied in detail. The reaction catalyses the conversion of one molecule of *o*-aminophenol to one molecule each of ammonia and catechol. The partially purified enzyme has a pH optimum of 6·2 in citrate–phosphate buffer and a temperature optimum of 45°. The metal ions, Mg^{2+}, Co^{2+} and Fe^{3+} were inhibitory to the reaction. Metal chelating agents like 8-hydroxyquinoline, *o*-phenanthroline, and diethyldithiocarbamate, caused a high degree of inhibition. A sulfhydryl requirement for the reaction was inferred from the inhibition of the reaction by *p*-chloromercuribenzoate and its reversal with GSH. Atebrin inhibition was reversed by addition of FAD to the reaction mixture.

INTRODUCTION

OUR previous investigations on the anthranilic acid oxidase system of *Tecoma stans* led to the resolution of this multienzyme system into three well-defined component enzymes.[1,2] The overall reaction catalysed by these component fractions is the oxidation of anthranilic acid to catechol via the intermediates 3-hydroxyanthranilic acid and *o*-aminophenol. After establishing the sequential events in the overall reaction, a detailed study was undertaken of the terminal step, i.e. the conversion of *o*-aminophenol to catechol, in order to obtain an insight into its mechanism. In this paper some of the properties of this reaction, with special reference to its stoichiometry, are presented.

RESULTS

Optimum Conditions for the Reaction

The optimum pH for the reaction was determined by incubating the reaction mixture with citrate–phosphate buffer at various pH values ranging from 3·4 to 7·4 (Fig. 1). The optimum pH was 6·2. The effect of concentration of the substrate on the enzyme reaction showed that activity increased linearly up to a final concentration of 5×10^{-4} M. Apparently high concentrations of substrate did not have any inhibitory effect on the reaction (Table 1). The rate of the enzyme reaction increased with temperature up to 45°, and then decreased up to 70° where there was complete destruction of enzyme activity.

Time Course and Stoichiometry

In order to get a clue to the mechanism of the reaction, the rate of the reaction at different time intervals was determined. These studies showed (Table 2) that about 64 per cent of

* C.S.I.R. Scientists' Pool Officer.

[1] P. M. NAIR and C. S. VAIDYANATHAN, *Phytochem.* 3, 235 (1964).
[2] P. M. NAIR and C. S. VAIDYANATHAN, *Phytochem.* 3, 513 (1964).

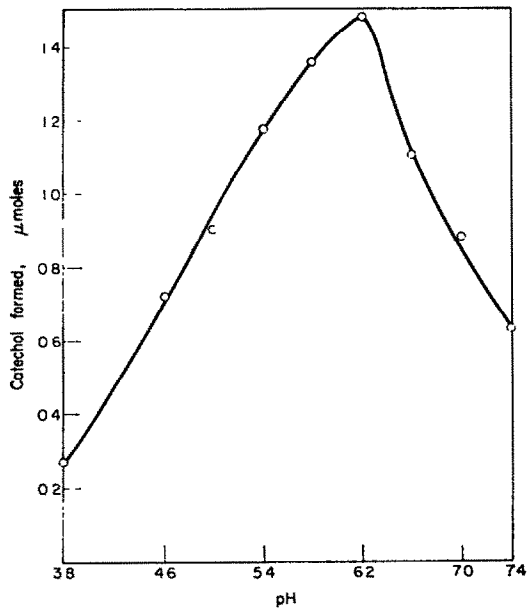

FIG. 1. EFFECT OF pH ON THE CONVERSION OF *o*-AMINOPHENOL TO CATECHOL BY *Tecoma stans*. Assay conditions were standard except for the use of 0·1 M citric acid–0·2 M disodium phosphate buffer of pH values between 3·4 and 7·4.

TABLE 1. EFFECT OF *o*-AMINOPHENOL CONCENTRATION ON ITS CONVERSION TO CATECHOL BY *Tecoma stans* ENZYME

Final concentration of *o*-aminophenol ($M \times 10^{-5}$)	Catechol formed (μmole)
1·0	0·019
2·5	0·048
5·0	0·098
10·0	0·180
25·0	0·468
50·0	0·917
100·0	1·472
200·0	1·482
300·0	1·480

Standard conditions of assay were employed except above-mentioned concentrations of *o*-aminophenol were added to the reaction mixture.

o-aminophenol was consumed in 15 min and after 30 min there was no further oxidation of *o*-aminophenol. At 30 min, there was no stoichiometry between the disappearance of *o*-aminophenol and the formation of catechol and ammonia. Beyond 30 min, however, although there was no further increase in the disappearance of substrate, there was appreciable

formation of the end products, viz. catechol and ammonia, and the rate of formation was linear with time up to 60 min. It is also interesting to note that at 60 min for every mole of *o*-aminophenol consumed there was concomitant formation of one mole each of ammonia and catechol.

TABLE 2. TIME COURSE AND STOICHIOMETRY OF *o*-AMINOPHENOL CONVERSION TO CATECHOL BY *Tecoma stans* ENZYME

Time (min)	*o*-Aminophenol consumed (μmole)	NH_3-formed (μmole)	Catechol formed (μmole)
15	1·045	0·395	0·401
30	1·640	0·948	0·979
45	1·640	1·598	1·578
60	1·640	1·638	1·635

The enzyme used in these experiments was Sup I. For NH_3 and catechol formation different reaction mixtures were used.

Effect of Metal Ions and Other Inhibitors

Some metal ions like Mg^{2+}, Co^{2+} and Zn^{2+} showed a differential inhibitory effect on the overall conversion of anthranilic acid to catechol[2]. Catechol formation was more susceptible to inhibition by these metal ions, as compared to the disappearance of anthranilic acid. The effect of these ions on the conversion of *o*-aminophenol to catechol showed that this reaction is highly susceptible to inhibition by them (Table 3). 8-Hydroxyquinoline, *o*-phenanthroline,

TABLE 3. EFFECT OF DIFFERENT SUBSTANCES ON CATECHOL FORMATION FROM *o*-AMINOPHENOL USING AN ENZYME SYSTEM FROM *Tecoma stans*

Compound added*	Inhibition (%)	Activation (%)
$HgCl_2$	21·0	
$MnSO_4$	0·0	
$MgSO_4$	40·0	
$ZnSO_4$	8·0	
$Co(NO_3)_2$	33·3	
$FeSO_4$	0·0	
$Fe_2(SO_4)_3$	33·3	
8-Hydroxyquinoline	80·0	
o-Phenanthroline	90·0	
DIECA	82·0	
EDTA	15·0	
Cyanide	40·0	
PCMB	64·0	
PCMB + GSH (10^{-3} M)		34·0
Atebrin	95·0	
Atebrin + FAD (10^{-3} M)		36·0
Hydroxylamine	0·0	

* The substances were added at a final concentration of 5×10^{-4} M.

and diethyldithiocarbamate (DIECA) were also highly inhibitory to this reaction. *p*-Chloromercuribenzoate (PCMB) and Atebrin inhibited the reaction and these effects were reversed by addition of GSH and FAD respectively to the reaction mixture (Table 3).

DISCUSSION

The conversion of *o*-aminophenol to catechol has been shown to be the terminal step in the enzymic oxidation of anthranilic acid to catechol by cell-free extracts of *Tecoma* leaves.[2] The results presented in this paper show that the properties of the enzyme catalysing this terminal step are different from those of the anthranilic oxidase system reported earlier, which catalysed the overall oxidation of anthranilic acid to catechol. However, there is a close similarity between the properties of this enzyme and those of the isophenoxazine synthase obtained from the same source.[3] Both the enzymes behave in an analogous manner to changes in the pH of the reaction and showed maximal activity at pH 6·2. The optimum temperature for both these enzymes was 45 .

A study of the rate of the reaction at different time intervals by assaying the disappearance of *o*-aminophenol and the formation of the products has helped to gain an insight into the possible mechanism of the reaction. The faster rate at which the substrate was consumed as compared to the rate of formation of the products, suggests the possibility of the existence of intermediate steps in the overall event. As in the case of isophenoxazine synthase which exhibits similar characteristics, it is probable that prior to the conversion to catechol, *o*-aminophenol might give rise to *o*-quinoneimine which on hydrolysis would yield *o*-benzoquinone, the reduction of which would lead to the formation of catechol. The hydrolysis of *o*-quinoneimine appears to be the rate-limiting step in the overall reaction. This is supported by the fact that strict stoichiometry between the disappearance of *o*-aminophenol and the formation of catechol and ammonia, was observed after prolonged incubation (Table 2).

From the foregoing it is quite probable that in the conversion of *o*-aminophenol to isophenoxazine as well as in the formation of catechol from the same substrate, the primary step might be the formation of *o*-quinoneimine. So a comparison of the properties of the two enzymes, viz. isophenoxazine synthase and the enzyme system catalysing the formation of catechol, is pertinent at this stage. Both the enzymes showed a remarkable similarity in their susceptibility to the action of various inhibitors. Atebrin at a concentration of 5×10^{-4} M inhibited both the reactions to the same extent and this inhibition was reversed by the addition

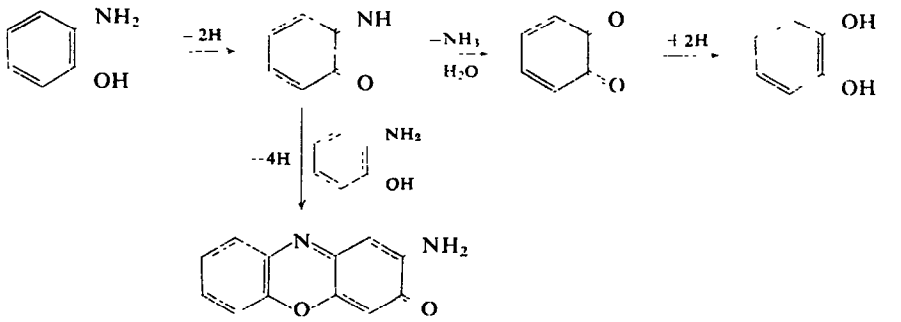

FIG. 2. THE TWO DIFFERENT PATHWAYS IN WHICH *o*-AMINOPHENOL CAN BE METABOLIZED IN *Tecoma stans* LEAVES.

P. M. NAIR and C. S. VAIDYANATHAN, *Biochim. Biophys. Acta* **81**, 507 (1964).

of FAD. Both the enzymes probably contain essential sulfhydryl groups as indicated by their inhibition by PCMB and the reversal of inhibition by GSH. Metal ions like Hg^{2+}, Mg^{2+} and Co^{2+} also inhibited these enzymes.

However, these two enzymes showed a marked difference in the optimal substrate concentration required by each (3×10^{-3} M in the case of isophenoxazine synthase and 10^{-3} M in the case of the enzyme catalysing the formation of catechol) and also in the effect of manganous ions. Mn^{2+} which activated isophenoxazine synthase had no effect on the enzyme catalysing the conversion of *o*-aminophenol to catechol.

The available evidence suggests that in the *Tecoma* plant, *o*-aminophenol can be metabolized by two different routes as illustrated in Fig. 2.

EXPERIMENTAL

Preparation of the Enzyme

Twelve grammes of mature *Tecoma stans* leaves were crushed in a mortar with 30 ml of cold distilled water. The extract was centrifuged at 12,000 *g* for 10 min. The clear supernatant obtained was adjusted to pH 3·0 with 12 N acetic acid (3·2 ml per 100 ml extract) and the precipitate formed removed by centrifugation at 12,000 *g* for 10 min. The residue was discarded. The supernatant was adjusted to pH 5·0 with 3 N NaOH. This was designated as Sup I.

To 30 ml of this supernatant 6·78 g of solid $(NH_4)_2SO_4$ were added. After 15 min, the precipitate formed was separated by centrifugation and discarded. To the supernatant an additional 4·59 g of solid $(NH_4)_2SO_4$ were added. The mixture was held in the cold for 15 min to allow complete precipitation. The precipitate was centrifuged out and dissolved in 30 ml glass distilled water and used as the enzyme. The NH_4 ions present in this enzyme extract did not have any effect on the reaction.

All operations were carried out between 0–4° unless otherwise stated.

Assay of the Enzyme Activity

The enzyme assay was performed in a reaction mixture consisting of 0·5 ml of (100 μmoles of phosphate and 50 μmoles of citrate) buffer, pH 6·2; 2 μmoles of *o*-aminophenol and 1 ml of enzyme in a final volume of 2 ml. The reaction mixture was incubated at 30° for 45 min. The reaction was stopped by the addition of 0·5 ml of 30% trichloroacetic acid and the volume was adjusted to 3 ml. After centrifugation to remove the coagulated proteins, 1 ml portion was taken for catechol determination by the method of Nair and Vaidyanathan.[4] Suitable controls were included in all assays performed.

o-Aminophenol disappearance was estimated using *p*-dimethylaminobenzaldehyde according to the procedure described for arylamines by Venkataraman *et al.*[5] Ammonia formation was determined using Conway microdiffusion method.[6]

[4] P. M. Nair and C. S. Vaidyanathan, *Anal. Biochem.* 7, 315 (1964).
[5] A. Venkataraman, P. R. Venkataraman and H. B. Lewis, *J. Biol. Chem.* 173, 641 (1948).
[6] E. J. Conway, *Microdiffusion Analysis and Volumetric Error*. Lockwood, London (1947).