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

STUDIES ON THE PROPERTIES OF A PURIFIED ANTHRANILIC ACID OXIDASE SYSTEM AND ITS SEPARATION INTO DIFFERENT ENZYMIC COMPONENTS

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Abstract—An enzyme system which catalysed the conversion of anthranilic acid to catechol has been purified 20-fold from a cell-free leaf extract of *Tecoma stans*. The optimum substrate concentration was 10^{-3} M and optimum temperature for the reaction was 45°. The presence of a multi-enzyme system was inferred from inhibition studies. The formation of catechol was inhibited by Mg^{2+}, Zn^{2+}, and Co^{2+} ions, whereas anthranilic acid disappearance was not affected to the same extent. The effect of metal chelating agents like EDTA, cyanide and pyrophosphate showed a similar trend. PCMB inhibited catechol formation but had no effect on anthranilic acid disappearance. The reaction was not inhibited by catalase, nor was it activated by peroxide-donating systems. This ruled out the possibility of peroxidative type of reaction. The overall reaction is markedly activated by NADPH and THFA.

This multi-enzyme was separated into three different components, by fractionation with Alumina C_γ and calcium phosphate gels. The overall reaction catalysed by these components can be represented as anthranilic acid → 3-hydroxy anthranilic acid → *o*-aminophenol → catechol.

INTRODUCTION

AFTER establishing the stoichiometry of the conversion of anthranilic acid to catechol by washed chloroplasts of *Tecoma stans* leaves,[1] efforts were made to obtain the enzyme system in a soluble form. Earlier attempts to solubilize the anthranilic acid oxidase system from *Pseudomonas* by Stanier and co-workers,[2,3] were unsuccessful because of the extreme lability of the enzyme. In fact, this step was the most unstable in the entire sequence of reactions from tryptophan to β-ketoadipic acid. Only those cells which had a very high endogenous respiration rate showed on solubilization a detectable anthranilic acid oxidase activity as determined by oxygen uptake measurements. Even in such cases, where the enzyme could be demonstrated in cell-free extracts, it was so unstable that it could not be purified further. So for nearly a decade this interesting enzyme system defied all attempts at purification and had perforce to remain almost in oblivion.

The possibility of obtaining the anthranilic acid oxidase system from *Tecoma stans* in a soluble form was therefore attempted. It was fairly stable in aqueous solution, and the only

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Abbreviations used—EDTA, ethylenediaminetetraacetate; NADPH, reduced nicotinamideadenine dinucleotidephosphate; FAD, flavinadeninedinucleotide; PCMB, *p*-chloromercuricbenzoate; Dieca, diethyldithiocarbamate; THFA, tetrahydrofolic acid; ATP, adenosinetriphosphate; GSH, glutathione (reduced).

[1] P. M. NAIR and C. S. VAIDYANATHAN, *Phytochemistry* **3**, 235 (1964).
[2] R. Y. STANIER and O. HAYAISHI, *J. Bact.* **62**, 367 (1951).
[3] R. Y. STANIER and O. HAYAISHI, *J. Bact.* **62**, 690 (1951).

difficulty encountered was the high catecholase activity exhibited by aqueous extracts. It was later observed that the anthranilic acid oxidase system could be readily separated by isoelectric precipitation. After separation of the two activities, anthranilic acid oxidase was further purified by fractional precipitation with ammonium sulfate. The data presented in Table 1 show that a 20-fold purification of the enzyme has been achieved. The percentage

TABLE 1. PROGRESS OF PURIFICATION OF ANTHRANILIC ACID OXIDASE

Step	Specific activity*	Total activity†	Times purified	Yield (%)
Crude enzyme	0·462	13·4	—	—
Supernatant I	1·000	13·00	2 1	97
Ammonium sulfate fraction (40–60%)	9 43	17·94	20·3	130

In all these estimations only anthranilic acid disappearance was estimated. The assay conditions were the same as described in "Experimental".

* mg anthranilic acid consumed per mg of protein under standard assay conditions.
† Anthranilic acid consumed (mg) per 100 ml of crude enzyme.

recovery of the enzyme in the final preparation which is about 130% indicates that some endogenous inhibitor of the enzyme has been removed during the process of purification.

Some of the general properties of the partially purified preparation were studied in order to get an insight into the mechanism of anthranilic acid oxidation in plants. The properties of the partially purified anthranilic acid oxidase are similar to those of the chloroplast enzyme system in all major respects.

Attention was next paid to separation of the different enzymic components of the anthranilic acid oxidase system. Adsorption studies with Alumina C_γ and calcium phosphate gels were successful in the separation of the individual steps involved in the reaction. There are three well-defined steps in the overall conversion of anthranilic acid to catechol. These steps are illustrated in Fig. 1.

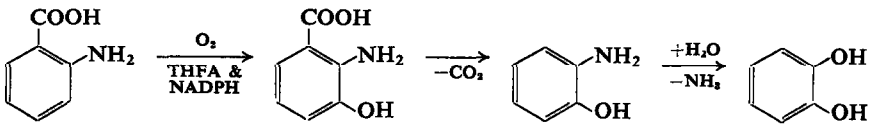

FIG. 1. THE SEQUENCE OF REACTIONS CATALYSED BY ANTHRANILIC ACID OXIDASE OF *Tecoma stans*.

RESULTS AND DISCUSSION

Optimum pH

The effect of pH on the activity of the purified anthranilic acid oxidase system was determined over the range 3·4 to 7·8 using citrate (0·1 M) and phosphate (0·2 M) buffers. As observed in the case of chloroplast preparation there was a sharp peak at pH 5·2 (Fig. 2). With further increase in pH the activity decreased and practically disappeared at pH 7·8. Stanier[3] observed a pH optimum of 7·5 for *Pseudomonas* anthranilic acid oxidase system using oxygen consumption as a measure of activity.

Effect of Substrate Concentration

In their response to increasing concentrations of substrate the activities of the chloroplast and purified enzyme preparations exhibited a striking contrast. The optimum substrate concentration for the chloroplast enzyme was 10^{-4} M. A further increase in the concentration of substrate brought about a marked lowering of activity and the reaction was completely

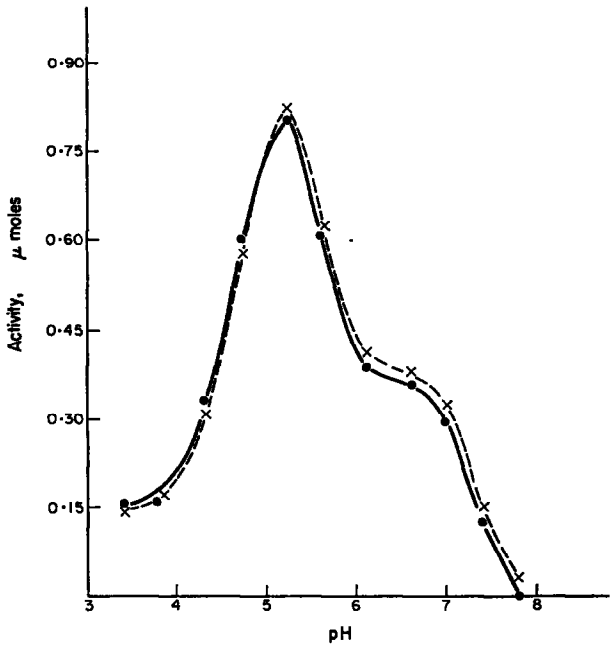

FIG. 2. EFFECT OF pH ON THE CONVERSION OF ANTHRANILIC ACID TO CATECHOL BY THE PARTIALLY PURIFIED ENZYME FROM *Tecoma stans* LEAVES.

Standard procedures described under "Experimental" were used.

———— Anthranilic acid consumed.
× — — — × Catechol formed.

inhibited at a concentration of 5×10^{-3} M. With the purified enzyme this effect was not observed, although at comparatively high substrate concentration of 2×10^{-3} M there was a slight depression of activity (Table 2). The optimum substrate concentration was 10^{-3} M for the purified enzyme.

Effect of Temperature

The results presented in Table 3 show that the enzyme had a maximal activity of 45°, but as there was a possible inactivation of enzyme due to protein denaturation, standard assays were always conducted at 30°.

Effect of Metal Ions and Other Inhibitors

The effect of various metal ions and other inhibitors was tried both with respect to anthranilic acid disappearance and catechol formation. The results given in Table 4 clearly show that formation of catechol was inhibited to an appreciable extent with Mg^{2+}, Zn^{2+} and Co^{2+}

TABLE 2. EFFECT OF SUBSTRATE CONCENTRATION

Final concentration of anthranilic acid ($M \times 10_5$)	Anthranilic acid consumed (μ moles)	Catechol formed (μ moles)
5	0·061	0·060
10	0·175	0·169
20	0·411	0·421
50	0·815	0 800
100	1·200	1·210
200	1·148	1 156

The time of incubation for this experiment was 30 min.

ions, whereas anthranilic acid disappearance was not affected to the same extent. From these observations, it can be surmised that a multi-enzyme reaction sequence is involved in the conversion of anthranilic acid to catechol. Similarly, catechol formation was more vulnerable to metal chelating agents like EDTA and cyanide, while some of the metal complexing agents like 8-hydroxyquinoline, *o*-phenanthroline, and Dieca were without effect on either of the two parameters. Pyrophosphate which also acts by complex formation with metal ions inhibited catechol formation, but had no effect on anthranilic acid disappearance. The selective nature of inhibition caused by PCMB was even more striking. According to the available evidence, the initial step in anthranilic acid oxidation was not inhibited by this reagent and that indicates that free sulfhydryl groups are not necessary for this step. The inhibition by PCMB of the formation of catechol which is the ultimate product of the multi-enzyme system indicates the involvement of sulfhydryl enzymes in some intermediate steps in the reaction chain. A strong inhibition of both anthranilic acid disappearance and catechol

TABLE 3. EFFECT OF TEMPERATURE

Temperature (°C)	Anthranilic acid disappeared (μ moles)	Catechol formed (μ moles)
25	0·800	0·757
30	0·847	0 810
37	0·897	0·891
45	0·950	0·921
55	0·821	0·800
60	0·700	0·696
70	0 510	0·527

The reaction mixtures were incubated at the above-mentioned temperatures for 10 min prior to the addition of substrate, in order to equilibrate. After that, the substrate was added and incubated at these temperatures for 30 min.

formation by atebrin and its complete reversal of FAD show that flavin enzymes are involved even in the first step of the reaction sequence. It has been suggested by Stanier[3] that hydroxylation of the aromatic ring of anthranilic acid is a prerequisite for its conversion to catechol. Katagiri *et al.*[4] recently reported a salicylate hydroxylase from soil bacteria which requires FAD for the hydroxylation and decarboxylation of salicylic acid. The observation on atebrin inhibition may indirectly indicate the possibility of flavin enzymes taking part in the multienzyme reaction.

TABLE 4. EFFECT OF METAL IONS AND OTHER INHIBITORS

Substance added*	Percentage inhibition	
	Anthranilic acid disappearance	Catechol formed
$MgSO_4$	15	48
$ZnSO_4$	21	52
$Co(NO_3)$	7	36
PCMB	0	60
Atebrin	66·6	100
Atebrin + FAD (10^{-3} M)	0	0
Hydroxylamine	42	40
Pyrophosphate	0	48
EDTA	34	48
Cyanide	20	54

* The final concentration of the various substances added in the reaction mixture was 5×10^{-4} M. $HgCl_2$, $MnSO_4$, $FeSO_4$, 8-hydroxyquinoline, *o*-phenanthroline, and Dieca did not have any effect on either parameter.

Effect of Ascorbic Acid and Nucleotide Coenzymes

Ascorbic acid is known to be an active participant in model hydroxylating systems. Udenfriend and co-workers[5,6] observed that non-specific, non-enzymic hydroxylation occurred in the presence of oxygen, ascorbic acid, ferrous iron and EDTA. The oxidation occurred over a wide pH range and it was found that ascorbic acid was used up during the reaction and dehydroascorbic acid was formed. Apart from this, NADPH has been reported to be an essential cofactor in a number of enzymatic hydroxylations.[7] Therefore, the effect of these compounds was tested on anthranilic acid oxidase system. Only NADPH activated the reaction to an appreciable extent. Neither FAD nor ascorbic acid had any effect (Table 5).

Effect of Tetrahydrofolic Acid

In recent years, evidence has been obtained to show that THFA is involved in enzymatic hydroxylation of phenylalanine to tyrosine.[8,9] Among a number of compounds tested,

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[7] H. S. MASON, *Adv. Enzymol.* **19**, 128 (1957).
[8] S. KAUFMAN, *Biochem. Biophys. Acta* **23**, 445 (1957).
[9] S. KAUFMAN, *J. Biol. Chem.* **226**, 511 (1957).

TABLE 5. EFFECT OF ASCORBIC ACID AND NUCLEOTIDE COENZYMES

pH of the reaction mixture	Substance added	Catechol formed (μ moles)	Anthranilic acid disappeared (μ moles)
5·2	None	0·900	0·930
	With FAD (10^{-3} M)	0·907	0·930
	With ascorbic acid (10^{-3} M)	0·899	0·930
6·2	None	0·370	0·395
	With NADPH	1·180	0·900

The enzyme used in this experiment was dialysed against water in the cold for 6 hr with stirring. In the case of NADPH, the reaction was conducted at pH 6·2 to facilitate the continuous regeneration of NADPH by enzymatic reduction. NADPH was produced *in situ* from NADP by coupling it with D-glucose-6-phosphate:NADP oxidoreductase. In experiments with ascorbic acid and FAD the reaction was done at pH 5·2 which is the optimum pH for anthranilic acid oxidase.

namely folic acid, ATP, GSH, THFA, only THFA was found to activate the anthranilic acid oxidase system. The data given in Table 6 show that there was a 60% increase in the amount of catechol formed when 0·2 μmole of THFA was added to the reaction mixture.

TABLE 6. EFFECT OF VARIOUS OTHER SUBSTANCES ON THE REACTION

Substance added	Catechol formed (μ moles)	Anthranilic acid disappeared (μ moles)
None	0·754	0·752
THFA	1·227	1·232
Folic acid	0·754	0·755
ATP	0·758	0·753
GSH	0 754	0·754

The enzyme used in this experiment was dialysed and only 0·5 ml of enzyme was used in the reaction mixture. The various substances were added 0·2 μmoles in 2-ml reaction mixture.

Conversion of Anthranilic Acid, 3-Hydroxyanthranilic Acid, o-Aminophenol, to Catechol

In order to find the intermediates in the overall reaction catalysed by anthranilic acid oxidase system, a number of probable intermediates in the reaction were used as substrate. It has been found (Table 7) that the crude enzyme (Sup. I, see Experimental) was capable of converting anthranilic acid, 3-hydroxyanthranilic acid and *o*-aminophenol to catechol, whereas pyrocatechuic acid, protocatechuic acid and salicylic acid remain unchanged.

TABLE 7. CATECHOL FORMATION FROM VARIOUS SUBSTRATES WITH ENZYME SUP. I

Substrates used	Catechol formed (μ moles)
Anthranilic acid	1·000
3-Hydroxyanthranilic acid	0·818
o-Aminophenol	1·281
Pyrocatechuic acid	None
Protocatechuic acid	None
Salicylic acid	None

The reaction mixture employed in this experiment was the same as mentioned under "Experimental" except that the above-mentioned substrates were used at a concentration of 2 μmoles instead of anthranilic acid. In the case of the last three substances in the table, chromatographic procedure was used to detect the catechol formation.

Chromatographic Identification of the Reaction Products Obtained on Incubating Anthranilic Acid and 3-Hydroxyanthranilic Acid with Sup. II, E_I and E_{II}

When Sup. I was treated with Alumina C_γ the enzyme which converts *o*-aminophenol to catechol was absorbed on the gel completely. The Sup. II obtained was able to convert both anthranilic acid and 3-hydroxyanthranilic acid to *o*-aminophenol. On treatment of Sup. II with calcium phosphate gel, the enzymes catalysing the reactions; anthranilic acid→3-hydroxyanthranilic acid and 3-hydroxyanthranilic acid→*o*-aminophenol were adsorbed on the gel. They were recovered from the gel by stepwise elution. In the presence of eluate I (E_I) anthranilic acid was oxidized to 3-hydroxyanthranilic acid, and incubation of 3-hydroxyanthranilic acid with eluate II (E_{II}) gave *o*-aminophenol. These products were identified by paper chromatography after isolation from the reaction mixture (Table 8).

Comparison with Other Hydroxylating Systems

It will be appropriate at this stage to compare the properties of the anthranilic acid oxidase system with those of other known hydroxylation systems. The present status of our knowledge about enzymatic hydroxylation reaction has been admirably reviewed by Mason[7] and Massart and Vercauteren.[10] In most cases the pattern of hydroxylation in the animal body is very similar to free radical hydroxylation. There are two versatile enzyme systems, viz, phenolase, also known as tyrosinase, which exhibits two activities, phenol-*o*-hydroxylase (cresolase) and catecholase activities, and peroxidase, which are implicated in aromatic hydroxylation reactions.

The *Tecoma* enzyme does not appear to be identical with either of the above enzymes for the following reasons: 1. It is resistant to the action of Dieca which is a specific inhibitor for copper enzymes like phenolase. 2. Purified phenolase with high cresolase activity was prepared from potato and was found to be incapable of oxidizing anthranilic acid. 3. *Tecoma* enzyme is not inhibited by catalase nor is it activated by peroxide-generating systems like L-leucine-L-amino acid oxidase and glucose-notatin.

[10] L. MASSART and R. VERCAUTEREN, *Ann. Rev. Biochem.* **28**, 527 (1959).

TABLE 8. CHROMATOGRAPHIC IDENTIFICATION OF THE REACTION PRODUCTS OF VARIOUS FRACTIONS Sup. II, E_I and E_{II}

Enzyme preparation used	Substrate used	Product of the reaction	R_f value* in different solvents		
			Solvent† A	Solvent B	Solvent C
Sup. II[1]	Anthranilic acid	*o*-Aminophenol		96	92
	3-Hydroxy-anthranilic acid	*o*-Aminophenol		96	92
E_I[2]	Anthranilic acid	3-Hydroxy-anthranilic acid	65		71
E_{II}[3]	3-Hydroxy-anthranilic acid	*o*-Aminophenol		96	92

* The R_f values of anthranilic samples of

(*a*) *o*-aminophenol in solvent B is 96 and in solvent C is 92.
(*b*) 3-Hydroxyanthranilic acid in solvent A is 65 and in solvent C is 71.

† Solvent A: $CHCl_3$–MeOH–H_2O–HCOOH, 250:25:24:1, v/v, upper phase.
Solvent B: Ethylmethylketone–acetone–H_2O–HCOOH, 40:2:7:1, v/v.
Solvent C: EtOH–conc. NH_3–H_2O, 18:1:1, v/v.

1. The reaction mixture contained 1 ml of citrate (0·1 M) phosphate (0·2 M) buffer pH 5·2. 0·5 ml anthranilic acid or 3-hydroxyanthranilic acid solution (10^{-2} M) and 2 ml of Sup. II. The reaction mixture was incubated for 1 hr at 30°. After the incubation period, it was deproteinized with 1ml 10 NH_2SO_4 and centrifuged to remove the precipitate. The supernatant was extracted with 10-ml quantities of ethyl acetate twice. The ethyl acetate layers were concentrated and the final volume was made up to 1 ml. A blank was also carried out under the same experimental conditions. 100 ml of this was spotted on the circumference of a small circle drawn at the centre of a Whatman No. 1 filter circle along with standard *o*-aminophenol solution. The bands were marked under u.v. and sprayed with diazotized sulfanilic acid.

2. The reaction mixture contained 2·5 ml of citrate–phosphate buffer pH 5·2, 0 5 ml anthranilic acid (10^{-2} M) solution and 5 ml of enzyme E_I. After incubation for 1 hr at 30°, the reaction was stopped by the addition of 10 NH_2SO_4 and extracted twice with 15 ml ethyl acetate. The layers were pooled and concentrated *in vacuo*. The chromatographic procedures were the same as above.

3. The composition of the reaction mixture was same as mentioned under footnote 2, except that hydroxyanthranilic acid and enzyme E_{II} were used.

In recent years, a large body of evidence has been accumulating which clearly underlines the importance of reduced pyridine nucleotides like NADH and NADPH as essential co-factors in certain enzymic hydroxylation reaction. The hydroxylation of steroids[11–15] and formation of 3-hydroxykynurenine from kynurenine[16,17] and conversion of phenylalanine to tyrosine[9,18,19] are cases in point.

The mechanism of aromatic hydroxylation has been a subject of much speculation. The

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[19] S. UDENFRIEND and J. R. COOPER, *J. Biol. Chem.* **194**, 503 (1952).

recent work of Kaufman[8,9,20,21] on the formation of tyrosine from phenylalanine has helped to give a new insight into the mechanisms of aromatic hydroxylation reactions. It has been found that during the hydroxylation reaction THFA was stoichiometrically oxidized to an intermediate. According to Kaufman,[22] the function of NADPH was to reduce the intermediate back to THFA. By virtue of its requirement for both NADPH and THFA (Tables 5 and 6), the anthranilic acid oxidase system of *Tecoma* is akin to the liver phenylalanine hydroxylase. The evidence obtained from the studies on the different enzymic components conclusively prove that the hydroxylation of anthranilic acid is the primary step in its conversion to catechol.

Although the occurrence of aromatic hydroxylation reactions in the animal systems has been extensively studied, the biosynthesis of polyphenols in plants has not received the kind of attention that their ubiquity demands. Hydroxylation reactions have recently been shown to take place *in vivo* by using radioactive tracer techniques. The amino acid phenylalanine has been found to be the most satisfactory precursor of several plant phenols. Geissman and Swain[23] found that *in vivo* phenylalanine-^{14}C was converted to radioactive caffeic acid by *N. tobacum* without rearrangement of the carbon skeleton. According to Neish,[24] phenylalanine may be first converted to phenyllactic acid then to cinnamic acid, but the direct conversion catalysed by phenylalanase is now considered more probable.[25] Cinnamic acid then undergoes stepwise hydroxylation to give various phenolic acids like *p*-coumaric, caffeic, ferulic and sinapic acids. It may, however, be pointed out that almost all the above investigations have been done with intact plants. The present investigation has therefore a special significance in that it represents one of the few reports of an aromatic hydroxylation reaction carried out by a cell-free enzyme system obtained from plant source. The only other reports of an *in vitro* system isolated from plant are that of Kaneko[26] who obtained anethole when a cell-free enzyme from the plant *Foeniculum vulgare* was incubated with phenylalanine and of Levy and Zucker[27] who demonstrated the formation of chlorogenic acid from cinnamyl and *p*-coumaryl esters of quinic acid by a cell-free enzyme system from potato.

EXPERIMENTAL

Cofactors

NADPH was prepared enzymatically by coupling D-glucose-6-phosphate: NADP oxidoreductase[28] and THFA was prepared by hydrogenation of folic acid according to the method of O'Dell *et al.*[29]

Purification of Anthranilic Acid Oxidase

Fresh, mature leaves of *Tecoma stans* (12 g) were crushed with a pestle and mortar and homogenized with 30 ml of cold distilled water. The homogenate was squeezed through

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cheese-cloth and the greenish extract was centrifuged at 12,000 *g* for 10 min in a refrigerated centrifuge. The supernatant (23 ml) was adjusted to pH 3·0 by careful addition of 6 N acetic acid and the resulting precipitate was removed by centrifugation at 12,000 *g* for 10 min. This precipitate contained catechol oxidase, and was discarded. The supernatant was adjusted to pH 5·0 with 3 N sodium hydroxide, and was designated as Sup. I.

To 20 ml of Sup. I, 4·52 g of solid ammonium sulfate were added in the cold (0–5°). After 15 min, the precipitate formed was removed by centrifugation in the cold. The precipitate thus obtained (at 0–40% saturation of ammonium sulfate) was inactive and was discarded. To the supernatant from the above, 3·06 g of solid ammonium sulfate were added. After 15 min the precipitate formed was separated by centrifugation, dissolved in 15 ml of water and the activity of this was determined without dialysis because it was found that ammonium salts at fairly high concentration did not have any effect on anthranilic acid oxidase activity. The final preparation was devoid of catecholase activity.

Ten ml of the above solution was dialysed exhaustively against distilled water with stirring to remove all traces of ammonium sulfate and the protein concentration of this solution was determined according to the method of Johnson.[30]

Preparation of Different Enzyme Components from Sup. I

Forty ml of Sup. I was treated with the residue obtained after centrifuging 30 ml of Alumina C_γ gel (25 mg/ml). This mixture was stirred in the cold for 30 min and the adsorbent was centrifuged out in the cold. The supernatant (Sup. II) was used as enzyme.

Thirty ml of Sup. II was treated with 14 ml of calcium phosphate gel (14 mg/ml). The mixture was stirred in the cold for 15 min and centrifuged. The supernatant was devoid of both activities. The enzymes were eluted differentially from the absorbant. The residue was suspended in 10 ml of 0·05 M sodium phosphate buffer pH 7·0. After 15 min standing in the cold, the residue was separated by centrifugation. The eluate was adjusted to pH 5·0 with dilute acetic acid, and used as enzyme E_I. The residue obtained after elution with 0·05 M phosphate buffer was again suspended in 10 ml of 0·1 M sodium phosphate buffer pH 7·0. The mixture was centrifuged after keeping in the cold for 15 min. The eluate was used as enzyme E_{II} after adjustment of pH to 5·0 with dilute acetic acid.

Enzyme Assay

The enzyme assay was performed in a reaction mixture containing 0·5 ml of citrate (0·1 M) and sodium phosphate (0·2 M) buffer pH 5·2, anthranilic acid 2 μmoles, and 1 ml of enzyme in a final volume of 2 ml. Unless otherwise stated, the reaction mixture was incubated at 30° for 30 min. Suitable controls were always included along with the assay tubes. In all experiments carried out with the partially purified enzyme both anthranilic acid disappearance and catechol formation were estimated to check whether these parameters varied *pari passu*, when the experimental conditions were changed. For the estimation of catechol and anthranilic acid, different reaction mixtures were used.

Catechol Estimation

At the end of the incubation period, the reaction was stopped by the addition of 0·5 ml of 30% trichloroacetic acid and 0·5 ml of formaldehyde (37–41%). After centrifugation to remove the precipitated protein, 1 ml aliquot was pipetted out and catechol estimated by a

[30] M. J. JOHNSON, *J. Biol. Chem.* **137**, 575 (1941).

colorimetric method by Nair and Vaidyanathan,[31] using sodium tungstate, sodium nitrite reagent.

Anthranilic Acid Estimation

For the estimation of anthranilic acid, the reaction was stopped by adding 0·5 ml of 30% trichloroacetic acid and 0·5 ml of water was added instead of formaldehyde. Anthranilic acid was determined in a 0·5 ml aliquot according to the method of Venkataraman *et al.*,[32] using Ehrlichs Reagent.

[31] P. M. NAIR and C. S. VAIDYANATHAN. *Anal. Biochem.* (In press).
[32] A. VENKATARAMAN, P. R. VENKATARAMAN and H. B. LEWIS, *J. Biol. Chem.* 173, 641 (1948).