Enzymic Conversion of 3-Hydroxyanthranilic Acid into Cinnabarinic Acid by the Nuclear Fraction of Rat Liver

By P. V. SUBBA RAO, N. S. JEGANNATHAN AND C. S. VAIDYANATHAN

Department of Biochemistry, Indian Institute of Science, Bangalore, India

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1. An enzyme solely localized in the nuclear fraction of rat liver was found to convert 3-hydroxyanthranilic acid into a red product that was isolated and crystallized from the reaction mixture. The product was identified as cinnabarinic acid (2-amino-3-oxo-3*H*-phenoxazine-1,9-dicarboxylic acid) by comparing its properties with synthetic cinnabarinic acid. 2. The enzyme had optimum pH at 7.2. Heavy-metal ions like Ag^+ , Hg^{2+} , MoO_4^{2-} , Fe^{2+} and Cu^{2+} were inhibitory; Mn^{2+} activated the reaction to a considerable extent. 3. The reaction was inhibited by mercaptoethanol, GSH and cysteine, and activated by *p*-hydroxymercuribenzoate and sodium arsenite, which may suggest the involvement of disulphide groups in the reaction.

The occurrence in Nature of a new class of organic pigments having a phenoxazine chromophore has been reported during recent years by several authors. These pigments are represented by ommochrome (Butenandt, Schiedt, Bieker & Cromartie, 1954), the actinomycins (Bullock & Johnson, 1957*a, b*) and certain mould metabolites like cinnabarin (Gripenberg, 1958; Cavill, Clezy, Tetaz & Werner, 1959). Matshoka (1960) has shown the presence of an isophenoxazine ring in questionmycin A isolated from *Streptomyces fungicidicus*.

Gripenberg (1958) and Cavill *et al.* (1959) isolated a red pigment, cinnabarin, from certain fungi and proposed the structure, 2-amino-9-hydroxymethyl-3-oxo-3*H*-phenoxazine-1-carboxylic acid. Along with cinnabarin, Gripenberg (1958) isolated cinnabarinic acid, formulated it as 2-amino-3-oxo-3*H*-phenoxazine-1,9-dicarboxylic acid and showed it to be identical with a compound synthesized by Butenandt, Keck & Neubert (1957) by the oxidation of 3-hydroxyanthranilic acid.

Radioisotope studies have indicated that tryptophan and some of its metabolites are involved in the biosynthesis of the phenoxazine ring. Butenandt & Beckmann (1955) and Butenandt & Neubert (1955) have shown that ommochromes like xanthommatin, rhodommatin and ommatin C possess radioactivity after the injection of either DL- $[\beta\text{-}^{14}\text{C}]$ tryptophan or DL- $[\delta\text{-}^{14}\text{C}]$ kynurenine. Sivak, Meloni, Nobili & Katz (1962) have shown that growing cultures of *Streptomyces antibioticus* preferentially incorporated the radioisotope of DL- $[\gamma\text{-}^{14}\text{C}]$ tryptophan into the phenoxazine chromophore of actinomycin.

More recently, Gutmann & Nagasawa (1959),

Katz & Weissbach (1962) and Madhusudanan Nair & Vaidyanathan (1964) have reported the enzymic synthesis of the phenoxazine ring. P. Madhusudanan Nair & L. C. Vining (personal communication) obtained an enzyme from spinach leaves that would convert 3-hydroxyanthranilic acid into cinnabarinic acid.

Viollier & Süllman (1950) reported the oxidation of 3-hydroxyanthranilic acid to a red product by a suspension of the sediment obtained after spinning a rat-liver homogenate at 3000 rev./min. These authors reported that no carbon dioxide or ammonia was liberated during the reaction and that there was a requirement for oxygen. The present paper represents an extension of our previous work on the metabolism of 3-hydroxyanthranilic acid (Subba Rao, Jegannathan & Vaidyanathan, 1964) and describes the characterization of the product of 3-hydroxyanthranilic acid by rat-liver homogenates as cinnabarinic acid. Some of the properties of the enzyme cinnabarinic synthase are reported.

MATERIALS AND METHODS

All reagents were of analytical grade and glass-distilled water was used for preparation of all solutions.

Chemicals. 3-Hydroxyanthranilic acid, *p*-hydroxymercuribenzoate, NAD, NADP, FMN and FAD were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. Arsenite, mercaptoethanol, cysteine and GSH were obtained from British Drug Houses Ltd., Poole, Dorset.

Paper chromatography. The following solvent systems were used for the chromatographic identification of cinnabarinic acid. Circular paper chromatograms were run at 30° on Whatman no. 1 filter paper with solvent A (collidine-

0.5M-KH₂PO₄; 2:3, v/v) or solvent *B* (collidine-lutidine-water; 1:1:2, by vol.). The organic layer was used in each case. For the separation of 3-hydroxyanthranilic acid from cinnabarinic acid, a third system, solvent *C* (ethyl methyl ketone-acetone-100% formic acid-water; 40:2:6:1, by vol.) was used.

Ultraviolet and visible spectra. A Beckman DB recording spectrophotometer was used for obtaining ultraviolet and visible spectra.

Infrared spectra. Infrared spectra were taken in a Perkin-Elmer Infracord spectrophotometer by using Nujol mulls.

Determination of protein. This was done by the method of Lowry, Rosebrough, Farr & Randall (1951). Crystalline bovine serum albumin was used as a standard.

Determination of cinnabarinic acid. Standard cinnabarinic acid solution was prepared in 0.01M-phosphate buffer (Na₂HPO₄-NaH₂PO₄), pH 7. Samples containing 3-40 µg. of cinnabarinic acid were diluted to 1 ml. with water followed by 2 ml. of ethanol, and the intensity of colour was measured in a Beckman DU spectrophotometer at 450 mµ. The colour intensity was linear up to 40 µg.

Determination of 3-hydroxyanthranilic acid. Since cinnabarinic acid interfered with the fluorescence of 3-hydroxyanthranilic acid, it was necessary to separate the latter from the former. The reaction mixture was extracted twice with equal volumes of peroxide-free ether after adjusting the pH to 2.5 with 0.5N-HCl. The ether extract was evaporated to dryness under suction and the residue was dissolved in ethanol. Suitable portions were spotted on a circular Whatman no. 1 filter paper and 3-hydroxyanthranilic acid was separated from cinnabarinic acid by using solvent *C*. 3-Hydroxyanthranilic acid moved with the solvent front, whereas cinnabarinic acid remained at the origin. The 3-hydroxyanthranilic acid band was marked under ultraviolet light, cut into pieces and eluted with ethanol, and the fluorescence was measured in a Klett fluorimeter according to the method of Long, Hill, Weinstock & Henderson (1954) with B₁ as primary filter and 385 mµ interference filter as secondary. A standard graph was prepared by using different concentrations of 3-hydroxyanthranilic acid under similar conditions.

Preparation of enzyme. Albino rats of either sex were killed under light ether anaesthesia by direct cardiac puncture and the liver was perfused with 0.9% NaCl. The perfused liver was weighed and cut into small pieces and homogenized in a Potter-Elvehjem homogenizer with thrice the weight of 0.9% NaCl at 0-4°. The liver homogenate was centrifuged at 50g for 2 min. The supernatant was centrifuged for 10 min. at 1000g in an International PR₂ refrigerated centrifuge at 0-4°. The nuclear fraction thus obtained (Schneider & Hogeboom, 1950) was washed twice with 0.9% NaCl and again centrifuged, and finally suspended in thrice the volume of 0.9% NaCl. The nuclear fraction was diluted 1:3 with 0.9% NaCl and used as enzyme.

Measurement of enzymic activity. The velocity of the reaction was followed by estimating the amount of cinnabarinic acid formed. The reaction mixture (1 ml.) contained sodium phosphate buffer, pH 7.2 (50 µmoles), 3-hydroxyanthranilic acid (2 µmoles) and enzyme (600 µg. of protein). After equilibration at 37°, the reaction was started by the addition of 0.2 ml. of enzyme. Nuclear fractions from rat liver were diluted with cold 0.9% NaCl before use to give a suitable concentration of protein. To test the formation of

cinnabarinic acid in the fraction obtained after the sedimentation of the nuclei, 0.5 ml. (containing about 200 mg. of protein) of the fraction was used. The reaction was stopped by the addition of 2 ml. of ethanol and centrifuged at 12000g for 10 min. Suitable samples of the clear supernatant were diluted to 3 ml. with 66% (v/v) ethanol, and the cinnabarinic acid formed was determined spectrophotometrically.

Isolation of the product of the reaction. The reaction mixture, containing 20 ml. of the nuclear fraction, 10 ml. of 0.2M-sodium phosphate buffer, pH 7.2, 30 mg. of 3-hydroxyanthranilic acid and water to give a final volume of 50 ml., was incubated for 6 hr. at 37°. After the specified time the reaction mixture was centrifuged at room temperature to remove the nuclei and the supernatant was repeatedly extracted with peroxide-free ether to remove 3-hydroxyanthranilic acid. The traces of 3-hydroxyanthranilic acid still remaining in the reaction mixture were removed by adjusting the pH to 6.5 with 1N-HCl and extracting repeatedly with ether. The reaction mixture, which was now completely free from 3-hydroxyanthranilic acid, was adjusted to pH 2.5 with 6N-HCl and extracted with peroxide-free ether, and evaporated to dryness after drying it over anhydrous Na₂SO₄. The residue was taken up in pyridine and crystallized, giving dark-reddish-brown crystals.

RESULTS

Identification of the enzymic reaction product. The properties of the enzymic product were compared with those of authentic cinnabarinic acid and found to be identical with it.

Melting point. Both cinnabarinic acid and the isolated product decomposed above 300° without melting.

Ultraviolet and visible spectra. Ethanol was used as the solvent. There was good agreement between the spectrum of the enzymic product and that of cinnabarinic acid. The absorption maxima were at 235, 430 and 450 mµ, and were identical with those reported by Butenandt *et al.* (1957).

Infrared spectrum. The infrared spectra of both the enzymic product and cinnabarinic acid were identical.

Chromatography. The *R_F* values of cinnabarinic acid and the enzymic product on paper chromatograms with solvents *A* and *B* were 0.55 and 0.7 respectively.

Intracellular distribution. Cinnabarinic acid-synthase activity was solely localized in the nuclear fraction. There was no cinnabarinic acid formation in the supernatant obtained after removing the nuclear fraction.

Optimum pH. Fig. 1 shows the effect of pH on the cinnabarinic acid-synthase activity, which was determined with 50 µmoles of sodium phosphate buffer ranging from pH 5.8 to 7.8. The pH optimum for the reaction was about 7.2.

Effect of substrate concentration. The effect of different concentrations of 3-hydroxyanthranilic

Fig. 1. Effect of pH on cinnabarinic acid formation in the presence of nuclear fraction of rat liver. The reaction mixture consisted of sodium phosphate buffer (50 μ moles), 3-hydroxyanthranilic acid (2 μ moles) and enzyme (600 μ g. of protein) in a final volume of 1 ml. After 30 min. incubation at 37°, the cinnabarinic acid formed was estimated as described in the Materials and Methods section. Specific activity is defined as μ mole of cinnabarinic acid formed/mg. of protein.

Fig. 3. Time-course of cinnabarinic acid formation by the nuclear fraction of rat liver. The conditions were as described in Fig. 1, except that the period of incubation was varied.

Fig. 2. Effect of substrate concentration on cinnabarinic acid formation by the nuclear fraction of rat liver. The conditions were as described in Fig. 1, except that the concentration of 3-hydroxyanthranilic acid was varied.

acid on the reaction is shown in Fig. 2. The optimum concentration was 10 μ moles (10mm final concn.). Higher substrate concentration had no inhibitory effect.

Table 1. *Stoichiometry of the conversion of 3-hydroxyanthranilic acid into cinnabarinic acid by the nuclear fraction of rat liver.*

The reaction mixture (1 ml.), containing sodium phosphate buffer, pH 7.2 (50 μ moles), 3-hydroxyanthranilic acid (2 μ moles) and enzyme (600 μ g.), was incubated at 37° for various periods. Cinnabarinic acid and 3-hydroxyanthranilic acid in the reaction mixture were estimated as described in the Materials and Methods section.

Time (min.)	3-Hydroxyanthranilic acid disappeared (μ moles)	Cinnabarinic acid formed (μ mole)
30	0.101	0.059
60	0.467	0.225
90	0.879	0.458
120	1.270	0.682

Stoichiometry and time-course of reaction. An initial lag up to 30 min. was observed, followed by a steady increase up to 120 min. (Fig. 3). There was a stoichiometric formation of 1 mol. of cinnabarinic acid for the disappearance of 2 mol. of 3-hydroxyanthranilic acid (Table 1).

Effect of enzyme concentration. Fig. 4 shows the effect of different amounts of enzyme on cinnabarinic acid formation. There was a steady increase

Fig. 4. Effect of enzyme concentration on the formation of cinnabarinic acid by the nuclear fraction of rat liver. The conditions were as described in Fig. 1, except that the concentration of enzyme was varied.

Table 2. Effect of different metal ions on cinnabarinic acid formation by the nuclear fraction of rat liver

Enzyme containing 600 μg. of protein was preincubated with the indicated final concentration of the compounds for 10 min. at 37° before the substrate was added. The rest of the procedure was as described in the Materials and Methods section.

Addition	Final concn. (mm)	Inhibition (%)	Activation (%)
CuSO ₄	0.05	93	—
	0.5	69	—
MnSO ₄	0.05	—	247
	0.5	—	172
	1.0	—	150
CaCl ₂	0.05	8	—
	0.5	5	—
AgNO ₃	0.5	89	—
HgCl ₂	0.5	100	—
ZnSO ₄	0.5	21	—
FeSO ₄	0.5	49	—
MgSO ₄	0.5	6	—
Na ₂ MoO ₄	0.5	34	—

in activity as the concentration of the enzyme was increased. In routine experiments 600 μg. of enzyme protein was used.

Influence of metal ions. The ions Ag⁺, Hg²⁺, MoO₄²⁻, Fe²⁺ and Cu²⁺ inhibited the reaction appreciably; Ca²⁺ and Mg²⁺ had no effect; Mn²⁺ activated the reaction to a considerable extent, and at a final concentration of 0.05 mm the increase in the reaction rate was as high as 247% (Table 2).

Table 3. Effect of different thiol inhibitors and thiol compounds on cinnabarinic acid formation by the nuclear fractions of rat liver

The conditions were as described in Table 2.

Addition	Final concn. (mm)	Inhibition (%)	Activation (%)
<i>p</i> -Hydroxymercuribenzoate	0.1	—	21
	0.5	—	17
Sodium arsenite	0.5	—	9
Mercaptoethanol	0.5	14	—
GSH	0.1	—	—
	1.0	40	—
Cysteine	0.2	100	—

Effect of the addition of different substances on the reaction. Metal-chelating agents like *o*-phenanthroline and EDTA caused no inhibition. Cyanide was a potent inhibitor. At a final concentration of 0.5 mm-cyanide there was about 75% inhibition. Diethyldithiocarbamate at a final concentration of 0.5 mm inhibited the reaction rate by about 37%.

p-Hydroxymercuribenzoate and arsenite activated the enzyme system (Table 3), but mercaptoethanol at a final concentration of 0.5 mm inhibited the reaction by about 40%. Cysteine at a final concentration of 0.2 mm inhibited the reaction completely. This suggests the possible involvement of a disulphide group of the enzyme protein in the reaction.

Atabrine (1 mm final concn.) had no effect on cinnabarinic acid synthase activity. Nucleotide cofactors like FMN, FAD, NAD and NADP did not activate the reaction.

DISCUSSION

Although several authors have implicated 3-hydroxyanthranilic acid as an intermediate in tryptophan-nicotinic acid conversion in animals, alternative pathways of this important metabolite have not been fully explored. Viollier & Süllman (1950) reported the formation of an unidentified red compound from 3-hydroxyanthranilic acid by the heavy particles obtained from rat-liver homogenates. The results of the present investigation clearly show that 3-hydroxyanthranilic acid is converted into cinnabarinic acid by rat-liver homogenates, and that the enzyme, cinnabarinic synthase (3-hydroxyanthranilic acid-oxygen oxidoreductase), is localized in the nuclear fraction of rat liver.

Though there are several reports on the chemistry and formation of phenoxazine compounds, the actual mechanism of the synthesis of the phenoxazine chromophore is not known. Butenandt, Biekert & Linzen (1956) and Butenandt (1957) suggested

Scheme 1. Suggested mechanism for the biosynthesis of cinnabarinic acid from 3-hydroxyanthranilic acid.

that xanthommatin and related pigments arise from 3-hydroxykynurenine by oxidative condensation in a manner similar to the chemical oxidation. Butenandt (1957) has suggested that 'dopaquinone' formed from dihydroxyphenylalanine by the action of tyrosinase can oxidize 3-hydroxykynurenine to xanthommatin *in vitro*.

Gutmann & Nagasawa (1959), Nagasawa, Gutmann & Morgan (1959), Katz & Weissbach (1962) and Madhusudanan Nair & Vaidyanathan (1964) have studied the enzymic oxidation of *o*-aminophenols to phenoxazine types of compound. From a study of the oxidation of various *o*-aminophenols by a mammalian cytochrome-cytochrome oxidase system, Nagasawa *et al.* (1959) concluded that *o*-quinoneimine is the primary product of the reaction and phenoxazine is formed by dimerization. The dimerization can occur only when the position *para* to the phenolic group is free.

Cinnabarinic acid synthase from rat liver had optimum pH at 7.2 (Fig. 3). This differs from the *Streptomyces* enzyme (Katz & Weissbach, 1962) and the phenoxazine synthase of plants (Madhusudanan Nair & Vaidyanathan, 1964), which had optimum pH values at 5.1 and 6.2 respectively.

Apparently no cofactors were required for the enzyme since externally added FMN, FAD, NAD and NADP had no effect. Atabrine also had no effect on the reaction.

On the basis of the results obtained, it may be suggested that the quinoneimine of 3-hydroxyanthranilic acid is first formed by dehydrogenation, and that this condenses with another molecule of 3-hydroxyanthranilic acid to give cinnabarinic acid. The condensation of the quinoneimine with 3-hydroxyanthranilic acid can occur either enzymically or non-enzymically. The reaction catalysed by cinnabarinic synthase can be depicted as shown in Scheme 1.

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