Enzymic Conversion of 3-Hydroxyanthranilic Acid into Cinnabarinic Acid

PARTIAL PURIFICATION AND PROPERTIES OF RAT-LIVER CINNABARINATE SYNTHASE

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Rat-liver cinnabarinic acid synthase (3-hydroxyanthranilic acid-oxygen oxidoreductase) was partially purified. Stoichiometric studies indicated the consumption of 3 atoms of oxygen/molecule of cinnabarinic acid formed. There was an initial lag in enzyme activity. The reaction had an optimum pH about 7.2 and an optimum temperature of 37°. The enzyme was highly specific for 3-hydroxyanthranilic acid. The system showed an absolute requirement for Mn^{2+} ions. Several bivalent metal ions and metal-chelating agents inhibited the reaction. Thiol inhibitors had no effect on enzyme activity, but reducing agents such as ascorbic acid were potent inhibitors. There was no requirement for any cofactor other than Mn^{2+} ions. The probable significance of the reaction in mammals is discussed.

Recent interest in the initiation of bladder tumours in experimental animals (Bryan, Brown & Price, 1964) by certain *o*-aminophenols such as 3-hydroxyanthranilic acid and 3-hydroxykynurenine, which are normal metabolites, as well as the excretion of abnormally large quantities of tryptophan metabolites in the urine of patients bearing bladder tumours in a normal population (Boyland & Williams, 1956; Brown, Price, Satter & Wear, 1960), calls for a fresh search for alternative pathways for the metabolism of these tryptophan metabolites. The enzymic conversion of 3-hydroxyanthranilic acid into cinnabarinic acid (2-amino-3-oxo-3*H*-phenoxazine-1,9-dicarboxylic acid) by the nuclear fraction of rat liver has been described by Subba Rao, Jegannathan & Vaidyanathan (1964). It was reported by Morgan & Weimorts (1964) that acetone-dried powders from livers of poikilothermic animals can also catalyse such an enzymic oxidation of 3-hydroxyanthranilic acid. This enzyme, which has been purified by Morgan, Weimorts & Aubert (1965), is unspecific and can oxidize related *o*-aminophenols also to phenoxazine compounds. In view of the carcinogenic activity of cinnabarinic acid (Boyland, 1960), further studies on the enzyme cinnabarinic acid synthase have been carried out. The enzyme from rat liver has been partially purified and properties of this enzyme, which is specific for 3-hydroxyanthranilic acid, are described.

MATERIALS AND METHODS

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

Chemicals. 3-Hydroxyanthranilic acid was purchased from Mann Research Laboratories Inc., New York, N.Y., U.S.A. Horse-heart cytochrome *c*, horse-radish peroxidase, ox-liver catalase, NAD, NADP, FMN, FAD and *p*-chloromercuribenzoate were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Sodium arsenite, 2,3-dimercaptopropanol, cysteine and GSH were obtained from British Drug Houses Ltd., Poole, Dorset. Haem and haematin were prepared from Sigma haemin.

Purification of enzyme. All operations were carried out at 0–4°.

Step 1. The nuclear fraction from 25 g. of rat liver was isolated according to the procedure of Subba Rao, Jegannathan & Vaidyanathan (1965). The nuclear fraction obtained from 25 g. of liver was finally suspended in 65 ml. of 0.9% NaCl.

Step 2. The nuclear fraction (65 ml.) was centrifuged at 12000 *g* for 5 min., the sediment was suspended in 60 ml. of 0.01 *M*-sodium phosphate buffer, pH 7.0, and homogenized in a VirTis homogenizer for 5–10 min. The extract was centrifuged at 12000 *g* for 10 min.

Step 3. To 54 ml. of solubilized extract from step 2 were added 6 ml. of 0.1 *M*- $MnSO_4$. The mixture was stirred for 15 min. and centrifuged at 12000 *g* for 15 min. The precipitate was discarded and the clear supernatant was dialysed thoroughly against water and clarified by centrifugation.

Step 4. The fraction from step 3 (50 ml.) was brought to 30% saturation by the addition of 8.2 g. of recrystallized $(NH_4)_2SO_4$, stirred for 15 min. and centrifuged at 12000 *g*

Table 1. *Purification of cinnabarinic acid synthase from rat liver*

The reaction mixture (1 ml.) containing sodium phosphate buffer, pH 7.2 (50 μ moles), 3-hydroxyanthranilic acid (4 μ moles), MnSO_4 (0.25 μ mole) and 0.1 ml. of enzyme was incubated at 37° for 30 min. (Preparations from steps 1–5 were diluted tenfold and the final preparation was diluted fivefold.) Cinnabarinic acid formed in the reaction mixture was estimated as described in the Materials and Methods section. A unit of activity is defined as the amount of enzyme that catalyses the formation of 1 μ mole of cinnabarinic acid/min.

Step	Volume (ml.)	Total protein (mg.)	Total activity (units)	Specific activity (units/mg. of protein)	Recovery (%)	Purification
1	65	845	112.2	0.13	100	—
2	60	366	66.0	0.18	58.9	1.4
3	62	149	59.5	0.41	53.1	3.2
4	41	31.2	33.2	1.06	29.6	8.2
5	27	6.75	22.5	3.30	20.0	25.4
6	54	2.38	20.3	8.53	18.1	65.6

for 15 min. The precipitate was discarded. The supernatant was brought to 60% saturation by the addition of 9.59 g. of $(\text{NH}_4)_2\text{SO}_4$. After stirring for 15 min., the precipitate was collected by centrifugation, suspended in 33 ml. of water and dialysed against water.

Step 5. The dialysed preparation (step 4) (30 ml.) was fractionated at -4° with acetone precooled to -20° . The precipitate obtained at 15–40% (v/v) acetone was collected, dissolved in 20 ml. of water and centrifuged to get a clear solution.

Step 6. The solution (15 ml.) of the precipitate from the acetone fractionation was adjusted to pH 6.0 with dilute acetic acid, and tricalcium phosphate gel (45 ml. of a preparation containing 14 mg./ml.; Keilin & Hartree, 1938) was added. The mixture was stirred for 15 min. and the gel was separated by centrifugation and resuspended in 30 ml. of 0.05 M-sodium phosphate buffer, pH 7.2. After 20 min. the mixture was centrifuged and the clear solution was used as enzyme.

The results of the purification procedure are given in Table 1.

Measurement of enzyme activity. The spectrum of cinnabarinic acid in 1% (w/v) trichloroacetic acid as taken on a Beckman model DB recording spectrophotometer is shown in Fig. 1. The absorption maxima were at 430 m μ and 450–452 m μ . The molar extinction coefficient (ϵ) at 450 m μ was 17280, and there was linearity between the extinction at 450 m μ and concentration of cinnabarinic acid in the range 5–40 μ g./3 ml.

The standard reaction mixture consisted of sodium phosphate buffer, pH 7.2 (50 μ moles), 3-hydroxyanthranilic acid (4 μ moles), MnSO_4 (0.25 μ mole) and 0.1 ml. of enzyme (0.8 μ g. of protein) in a total volume of 1 ml. After incubation for 30 min. at 37°, the reaction was stopped by the addition of 1 ml. of 3% (w/v) trichloroacetic acid followed by 1 ml. of water. After centrifugation, 1 ml. samples were diluted to 3 ml. with 1% (w/v) trichloroacetic acid, and cinnabarinic acid formed was calculated from the extinction at 450 m μ . To correct for non-enzymic oxidation of 3-hydroxyanthranilic acid, blanks containing boiled enzyme were set up in each experiment.

Determination of protein. This was done according to the method of Lowry, Rosebrough, Farr & Randall (1951).

Fig. 1. Visible spectrum of cinnabarinic acid (6.2 μ g./ml.) in an aqueous solution of 1% (w/v) trichloroacetic acid.

Oxygen uptake. Warburg manometers contained sodium phosphate buffer, pH 7.2 (100 μ moles), 3-hydroxyanthranilic acid (6 μ moles), MnSO_4 (0.75 μ mole) and 1 ml. of enzyme in a final volume of 3 ml. The reaction was started by the addition of substrate from the side arm and the oxygen uptake was measured over a period of 60 min. at 30°.

Spectrophotometry. The reduction and reoxidation of cytochrome *c* in the presence of 3-hydroxyanthranilic acid and the enzyme was measured in cuvettes maintained at 30°. Difference spectra were recorded on a Beckman model DB recording spectrophotometer. The reaction mixture (3.0 ml.) consisted of sodium phosphate buffer, pH 7.2 (100 μ moles), cytochrome *c* (0.2 μ mole), MnSO_4 (0.3 μ mole) and 3-hydroxyanthranilic acid (0.1 μ mole). It was scanned against a blank in which the substrate was omitted. After the addition of 0.5 ml. of enzyme to both cuvettes, the spectrum was recorded at 5 min. intervals.

RESULTS

Requirement for Mn^{2+} ions. The partially purified enzyme showed an absolute requirement for Mn^{2+}

Table 2. *Effect of Mn²⁺ ions on cinnabarinic acid formation by purified rat-liver cinnabarinic synthase*

Enzyme containing 0.8 μ g. of protein was preincubated with the indicated final concentration of MnSO₄ for 5 min. at 37° before the substrate was added. The rest of the procedure was as described in the Materials and Methods section.

Concn. of MnSO ₄ (mm)	Cinnabarinic acid formed (μ mole)
0	0
0.01	0.05
0.025	0.09
0.05	0.12
0.1	0.15
0.25	0.18
0.5	0.12
1.0	0.10

ions, the optimum concentration of which was 0.25 mm in the presence of 4 mm 3-hydroxyanthranilic acid (Table 2). None of the other bivalent metal ions Cu²⁺, Fe²⁺, Ca²⁺, Mg²⁺ and Zn²⁺ were effective. The enzyme preparations retained activity for at least 10 days without appreciable loss when stored at 4°.

Optimum pH. The optimum pH for purified cinnabarinic synthase from rat liver was about 7.2 when determined with sodium phosphate buffers ranging from pH 6.2 to 7.8.

Substrate concentration and substrate specificity. The optimum substrate concentration for the reaction was 4 mm. The K_m calculated from Lineweaver-Burk plots was 1.25×10^{-3} M. The enzyme showed a remarkably high specificity for 3-hydroxyanthranilic acid. The structurally related compounds 3-hydroxykynurenine and *o*-aminophenol were not oxidized when tested under standard assay conditions.

Time-course of the reaction. As observed with the nuclear fraction (Subba Rao *et al.* 1965), the formation of cinnabarinic acid mediated by the purified enzyme exhibited a lag phase (Fig. 2). The lag period could not be abolished by preincubating the enzyme with Mn²⁺ ions for 10 min. In exhibiting an initial lag in enzyme activity, rat-liver cinnabarinic synthase resembles tryptophan pyrrolase (Tokuyama & Knox, 1964). However, preincubation of the purified enzyme with haem (0.5 μ mole) or haematin (0.5 μ mole) plus ascorbic acid (0.5 μ mole) or sodium borohydride (0.5 μ mole) did not abolish the lag period. In fact, these reducing agents inhibited the reaction completely (Table 3).

Oxygen uptake. In the course of the reaction catalysed by cinnabarinic synthase 1.8 μ g. atoms of oxygen were taken up for the formation of 0.63 μ mole of cinnabarinic acid in 1 hr.

Fig. 2. Time-course of cinnabarinic acid formation in the presence of purified rat-liver cinnabarinic synthase. The reaction mixture consisted of sodium phosphate buffer (50 μ moles), 3-hydroxyanthranilic acid (4 μ moles), MnSO₄ (0.25 μ mole) and enzyme (0.8 μ g. of protein) in a final volume of 1 ml. The incubation was at 37° for various periods. Cinnabarinic acid formed was estimated as described in the Materials and Methods section.

Table 3. *Effect of reducing agents on the activity of purified rat-liver cinnabarinic synthase*

The conditions were as described in the Materials and Methods section.

Addition	Final concn. (mm)	Inhibition (%)
GSH	0.5	21
	1.0	58
Cysteine	0.5	0
	1.0	68
2,3-Dimercaptopropanol	0.5	100
Ascorbic acid	0.1	53
	0.5	100
Sodium borohydride	2 mg./ml.	100

Influence of temperature. The enzyme showed maximal activity at 37°. Further increase in temperature caused rapid loss in activity. The enzyme was inactivated when heated at 60° for 5 min. in the absence of substrate.

Effect of metal ions. These effects, which were determined in the presence of optimum concentration of Mn²⁺ ions, are summarized in Table 4. The ions Cu²⁺, Hg²⁺, Ag⁺, Fe²⁺, Fe³⁺, Ni²⁺ and MoO₄²⁻ caused appreciable inhibition. Enzyme activity was not affected by Ca²⁺, Mg²⁺ and Zn²⁺ ions.

Table 4. *Effect of metal ions on the activity of purified rat-liver cinnabarinic acid synthase*

The reaction mixture containing sodium phosphate buffer, pH 7.2 (50 μ moles), MnSO_4 (0.25 μ mole) and 0.1 ml. of enzyme (0.8 μ g. of protein) was preincubated for 10 min. with the indicated metal ion (final concn. 0.5 mM) before the addition of 3-hydroxyanthranilic acid (4 μ moles). The rest of the conditions were as described in the Materials and Methods section.

Addition	Inhibition (%)
AgNO_3	95
CdCl_2	5
NiSO_4	64
CaCl_2	0
MgSO_4	5
CuSO_4	68
FeSO_4	36
FeCl_3	27
ZnSO_4	9
Na_2MoO_4	100
CoCl_2	18
HgCl_2	91

Table 5. *Effect of metal-binding agents on the activity of purified rat-liver cinnabarinic acid synthase*

The conditions were as described in Table 4.

Addition	Final concn. (mM)	Inhibition (%)
Sodium azide	0.5	68
	1.0	77
Diethyldithiocarbamate	0.5	59
	1.0	64
<i>o</i> -Phenanthroline	1.0	—
$\alpha\alpha'$ -Bipyridyl	1.0	—
EDTA	0.5	9
	1.0	86
Potassium cyanide	0.5	73

Effect of other substances on the reaction. The metal-binding agents EDTA, diethyldithiocarbamate, cyanide and sodium azide inhibited the reaction to a considerable extent (Table 5). *o*-Phenanthroline and $\alpha\alpha'$ -bipyridyl had no inhibitory effect.

The reaction was not inhibited by the thiol inhibitors *p*-chloromercuribenzoate, *N*-ethylmaleimide, sodium arsenite and iodoacetic acid at concentrations varying from 0.1 to 1 mM.

The electron acceptors NAD, NADP, FMN, FAD and menadione had no effect on the enzymic formation of cinnabarinic acid, but 0.01 mM-cytochrome *c* inhibited the reaction by 20%. Atabrine (1 mM) had no effect on enzyme activity.

Spectrophotometric studies. Examination of absorption spectra showed that cytochrome *c* was immediately reduced by 3-hydroxyanthranilic acid. Nagasawa & Gutmann (1959) reported that cytochrome *c* is reduced by several *o*-aminophenols. When a reaction mixture (3.0 ml.) of sodium phosphate buffer, pH 7.2 (100 μ moles), cytochrome *c* (1 μ mole) and 3-hydroxyanthranilic acid (0.1 μ mole) was incubated for 30 min. at 37°, a yellow product was formed which was extractable into ether at pH 2.5. This was identified as cinnabarinic acid by comparing its absorption spectrum in ethanol and R_F values on paper chromatograms with those of an authentic sample (Subba Rao *et al.* 1965). However, under our experimental conditions, the cytochrome *c* reduced by 3-hydroxyanthranilic acid was not reoxidized by rat-liver cinnabarinic acid synthase. Further, cytochrome *c* prepared by reduction with sodium borohydride was not reoxidized by the enzyme preparation in the absence of 3-hydroxyanthranilic acid or any other reducing agent.

Effect of substrate analogues. Anthranilic acid, 3-hydroxykynurenine, *o*-aminophenol and *o*-, *m*- and *p*-hydroxybenzoic acid had no effect on the reaction. Catechol and *p*-aminobenzoic acid inhibited the reaction by about 20% at a final concentration of 0.5 mM. Lack of activation by catechol of cinnabarinic acid formation indicates that catechol oxidase (or polyphenol oxidase) is not responsible for the formation of cinnabarinic acid from 3-hydroxyanthranilic acid.

Influence of hydrogen peroxide and catalase. Hydrogen peroxide (final concn. 0.1%) neither removed the initial lag phase (Fig. 2) nor activated the reaction. Catalase (1 mg.) had no effect on enzyme activity. These results suggest that hydrogen peroxide is not formed during the oxidation of 3-hydroxyanthranilic acid.

DISCUSSION

The results presented in this paper show that the enzyme from rat-liver nuclei which catalyses the oxidation of 3-hydroxyanthranilic acid to cinnabarinic acid (2-amino-3-oxo-3*H*-phenoxazine-1,9-dicarboxylic acid) can be extensively purified. The optimum pH of 7.2 for rat-liver cinnabarinic acid synthase is similar to that reported for liver preparations from poikilothermic animals by Morgan *et al.* (1965). However, it is different from *Streptomyces* (Katz & Weissbach, 1962) and spinach (Nair & Vining, 1964) enzymes, which showed maximal activity at pH 5.1 and 9.0 respectively.

The system apparently showed no requirement for any cofactor other than Mn^{2+} ions. The inability of the nuclear cinnabarinic acid synthase from rat liver to reoxidize reduced cytochrome *c* in the

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

presence of 3-hydroxyanthranilic acid rules out the possibility of a system analogous to the mitochondrial cytochrome *c*-cytochrome oxidase system (Nagasawa & Gutmann, 1959; Nagasawa, Gutmann & Morgan, 1959).

The overall reaction catalysed by cinnabarinic synthase involves the conversion of 2 molecules of 3-hydroxyanthranilic acid into 1 molecule of cinnabarinic acid (Subba Rao *et al.* 1965) with the consumption of 3 atoms of oxygen. Such an oxidative dimerization may involve an intermediary formation of the *o*-quinoneimine of 3-hydroxyanthranilic acid. As suggested by Morgan *et al.* (1965), the highly reactive transient *o*-quinoneimine (II) can condense in two ways, namely either with another molecule of *o*-quinoneimine (II) or with a molecule of 3-hydroxyanthranilic acid (I), as depicted in Scheme 1. Cinnabarinic acid (VII) is the final product of both pathways. The initial lag in the formation of cinnabarinic acid, as well as the extreme sensitivity of the enzyme to reducing agents, suggests that an intermediate quinoneimine is involved in the reaction.

It is noteworthy that Ehrensvärd, Liljekvist & Heath (1960) observed that compounds (IV) and (VII) (Scheme 1) are formed during the oxidation of 3-hydroxyanthranilic acid by human serum, the process being activated by Cu²⁺ or Mn²⁺ ions. They suggested that this enzymic formation of cinnabarinic acid (VII), which was cyanide- and azide-sensitive, accounts for 15% of the 3-hydroxyanthranilic acid (I) that is oxidized. They assume that the rest of the 3-hydroxyanthranilic acid underwent a non-specific oxidation to compound (IV) catalysed by metal-protein complexes. In our experiments also the formation of cinnabarinic acid by rat-liver cinnabarinic synthase is strongly inhibited by cyanide and azide.

3-Hydroxyanthranilic acid and 3-hydroxykynurenine are known to induce neoplasms under certain conditions. Allen, Boyland, Dukes, Horning & Watson (1957), and more recently Bryan *et al.* (1964), have unequivocally established that these two normal metabolites can induce neoplasms locally when implanted into the bladder of mice. It has been repeatedly emphasized that the binding

of carcinogens to proteins is a prerequisite for their carcinogenic activity. Gutmann, Peters & Burtle (1956) suggested that a carcinogen can be covalently bound to protein if the former is converted into a highly reactive intermediate such as a quinone-imine. Support for the formation of such intermediates with quinonoid structure was provided by Nagasawa & Gutmann (1959), who demonstrated that the mammalian cytochrome *c*-cytochrome oxidase system can catalyse the oxidation of carcinogenic *o*-aminophenols to reactive quinone-imines. However, in contrast with the cytochrome *c*-cytochrome oxidase system and with the liver enzyme from poikilothermic animals (Morgan *et al.* 1965), rat-liver cinnabarin synthase is highly specific for 3-hydroxyanthranilic acid. This may mean that this enzyme is significant in the aetiology of tumours since it is involved in the metabolism of a known carcinogen, namely 3-hydroxyanthranilic acid.

Schultz (1962) postulated that mammalian cancer may be a manifestation of deranged tryptophan metabolism that may lead to the production of an endogenous carcinogen via the condensation of 3-hydroxykynurenine and *o*-aminophenol. Since it is known that tryptophan metabolites such as 3-hydroxyanthranilic acid and 3-hydroxykynurenine are excreted in abnormally high proportions by patients bearing bladder tumours (Boyland & Williams, 1956; Brown *et al.* 1960), it seems reasonable to assume that deranged tryptophan metabolism may lead to the maintenance of an abnormally high concentration of 3-hydroxyanthranilic acid in the system. This in turn may lead to the formation of cinnabarinic acid by cinnabarin synthase. The finding (Boyland, 1960) that the phenoxazine dimer of 3-hydroxyanthranilic acid produces significant incidence of bladder tumours in experimental animals lends further support to this view.

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