THE CONVERSION OF 3-HYDROXYANTHRANILIC ACID TO CINNABARINIC ACID BY THE NUCLEAR FRACTION OF RAT LIVER

P.V. Subba Rao, N.S. Jegannathan and C.S. Vaidyanathan
Department of Biochemistry, Indian Institute of Science, Bangalore, India.

Received April 27, 1964

Although several authors have implicated 3-hydroxyanthranilic acid (3-OHA) as an intermediate in tryptophan-niacin pathway in animals (Kaplan, 1961), alternative pathways of metabolism of this compound have not been fully explored. Madhusudanan Nair obtained an enzyme from spinach leaves which could convert 3-OHA to cinnabarinic acid (private communication). Viollier and Süllmann (1950) reported the conversion of 3-OHA to an unidentified red compound by rat liver homogenates. The present investigation describes the identification of this product as cinnabarinic acid (2-amino-3-H-isophenoxazine-3-one-1,9-dicarboxylic acid). Cinnabarinic acid is known to occur in nature along with cinnabarin isolated from the fungus Polystictus sanguineus (Gripenberg et al., 1957; Gripenberg, 1958).

EXPERIMENTAL

Albino rats were killed and the liver was perfused with 0.9% saline. The perfused liver was homogenized in a Potter-Elvehjem homogenizer with thrice the volume of 0.9% saline at 0-4°. The liver homogenate was centrifuged at 50xg

for 2 min. The supernatant was centrifuged for 10 min. at 1000xg in the cold (0-4°). The nuclear fraction thus obtained (Schneider and Hogeboom, 1950) was washed twice with 0.9% saline, again centrifuged and finally suspended in thrice the volume of 0.9% saline. The nuclear fraction was diluted in the ratio of 1:3 with 0.9% saline and used as enzyme.

Protein was determined by the method of Lowry et al. (1951). Cinnabarinic acid was determined by measuring the optical density at 450 mμ in a Beckman DU spectrophotometer after stopping the reaction with 2 ml. ethanol.

The velocity of the reaction was followed by measuring the amount of cinnabarinic acid formed.

The reaction mixture (1 ml.) contained 0.1 M phosphate buffer, pH 7.2 (0.5 ml.), 3-OHA (2 μmoles) and enzyme (600 μg. protein). The reaction tubes were incubated at 37°. Suitable controls were included.

Isolation of the product of the reaction:- The reaction mixture containing 20 ml. enzyme, 10 ml. 0.2 M phosphate buffer (pH 7.2), 30 mg. of 3-OHA and water in a final volume of 50 ml. was incubated for 6 hrs. at 37°. 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-OHA. The traces of 3-OHA still remaining were removed by adjusting the pH to 6.5 and extracting repeatedly with ether. The reaction mixture which was completely free from 3-OHA was adjusted to pH 2.5 and extracted with peroxide-free ether, and taken to dryness after shaking with anhydrous sodium sulphate. The residue

was taken in pyridine and crystallized giving dark reddish brown crystals.

RESULTS AND DISCUSSION

Identification of the enzymatic reaction product:- The properties of the enzymatic reaction product were compared with those of synthetic cinnabarinic acid obtained from Prof. Butenandt.

Like cinnabarinic acid, the isolated product decomposed above 300° without melting. Both cinnabarinic acid and enzymatic product showed absorption maxima at 235, 430 and 450 mμ in ethanol and these were identical with those reported by Butenandt et al. (1957). Infrared spectra of both synthetic cinnabarinic acid and enzymatic product were identical. In collidine - 0.5 M KH_2PO_4 (2:3) (organic phase) both compounds moved with an R_f of 0.55 on a circular Whatman No.1 filter paper.

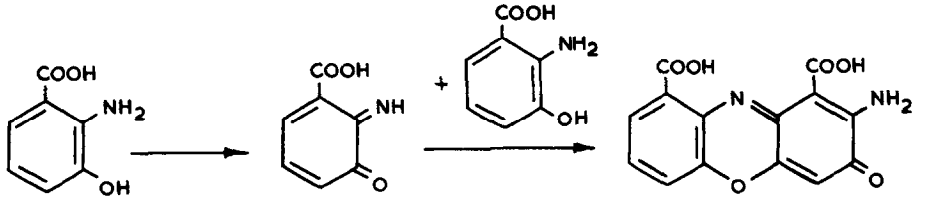
Table I shows the effect of time on cinnabarinic acid formation. An initial lag upto 30 min. was observed followed by a steady increase upto 120 min.

TABLE I

Time course of formation of cinnabarinic acid from 3-OHA in the presence of rat liver nuclei

Time in min.	umoles of cinnabarinic acid formed/mg. protein
15	0.01
30	0.02
45	0.05
60	0.08
90	0.17
120	0.24
180	0.27

Though there are several reports on the chemistry and formation of compounds having an isophenoxazine chromophore, the actual mechanism of the synthesis of isophenoxazine nucleus is not known. Butenandt (1954, 1957) suggested that xanthommatin and related pigments arise from 3-hydroxykynurenine by oxidative condensation of 2 molecules in a manner similar to the chemical oxidation. In analogy with the above mechanism, as well as similar work on related systems (Gutman et al., 1959; Nagasawa et al., 1959; Katz and Weissbach, 1962), it may be suggested that quinoneimine of 3-OHA is formed as an intermediate by dehydrogenation which then condenses with another molecule of 3-OHA to give cinnabarinic acid. The initial lag in the formation of cinnabarinic acid is consistent with this hypothesis. The reaction may be represented as follows:



ACKNOWLEDGEMENTS

We are very grateful to Prof. A. Butenandt for his generous gift of cinnabarinic acid and Prof. P.S. Sarma for his keen interest.

REFERENCES

Butenandt, A., U. Schiedt, E. Biekert and R.J.T. Cromartie, Ann. Chem. Liebigs, 590, 75 (1954).
Butenandt, A., Angew. Chem., 69, 16 (1957).
Butenandt, A., J. Keck and G. Neubert, Ann. Chem. Liebigs, 602, 61 (1957).

Gripenberg, J., E. Honkanen and O. Patoharju, Acta. Chem. Scand., 11, 1485 (1957).
Gripenberg, J., Acta. Chem. Scand., 12, 603 (1958).
Gutman, H.R. and H.T. Nagasawa, J. Biol. Chem., 234, 1593 (1959).
Kaplan, N.O., in Metabolic Pathways, Vol. 2, edited by D.M. Greenberg (Academic Press, New York), pp.634 (1961).
Katz, E. and H. Weissbach, J. Biol. Chem., 237, 882 (1962).
Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randell, J. Biol. Chem., 193, 265 (1951).
Nagasawa, H.T., H.R. Gutman and M.R. Morgan, J. Biol. Chem., 234, 1600 (1959).
Schneider, W.C. and G.H. Hogeboom, J. Biol. Chem., 183, 123 (1950).
Viollier, G.S. and H. Süllmann, Helv. Chim. Acta, 33, 776 (1950).