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Note

Improved method for the estimation of tryptophan hydroxylase activity using high-performance liquid chromatography with electrochemical detection

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Tryptophan hydroxylase (tryptophan 5-monooxygenase, E.C. 1.14.16.4) catalyses the conversion of L-tryptophan (TRP) to 5-hydroxytryptophan (5-HTP)[1,2]. In vertebrates this enzyme is believed to be the rate-limiting step in the biosynthesis of 5-hydroxytryptamine (serotonin, 5-HT)[3]. As tryptophan hydroxylase possesses low activity and is unstable, examination of it has proved difficult. In the past fluorimetric and radioenzymatic methods have been used to determine its activity and characteristics[4–7]. Both methods have limitations[8]. The fluorimetric method is relatively non-specific and insensitive. It can be quenched by high levels of TRP and assumes that no conversion of 5-HTP to 5-HT occurs. The radioenzymatic method, while very sensitive, relies on the conversion of radiolabeled TRP to 5-HTP and the subsequent conversion of 5-HTP to 5-HT, which is then extracted and measured. This assumes that there is an active aromatic amino acid decarboxylase present and that all 5-HTP produced is converted to 5-HT. Furthermore, the continuing metabolism of 5-HT by monoamine oxidase, N-acetyltransferase and other enzymes must be completely inhibited. Many tissues, such as pineal gland[9] and insect nervous tissue[10,11], possess high levels of N-acetyltransferase activity and this may affect estimates of tryptophan hydroxylase activity as derived from radioenzymatic assays.

Recently high-performance liquid chromatography (HPLC) with electrochemical detection has been used to determine tryptophan hydroxylase activity[12,13]. The separation of the metabolites 5-HT, 5-hydroxyindoleacetic acid (5-HIAA) and N-acetyl-5-hydroxytryptamine (NA-5-HT) and their possible contribution to the assay system however were not discussed; furthermore, detection limits for 5-HTP in the method appear to be only in the range of 10^{-9}–10^{-10} g.

This present report describes a more sensitive procedure (10^{-11} g) for the direct determination of 5-HTP produced by the hydroxylation of TRP. The method is also able to detect 5-HT, 5-HIAA and NA-5-HT if they are present in the incubate. The method has been applied to the determination of the activity of, and some characteristics of, tryptophan hydroxylase in rat and cockroach nervous tissues. The results obtained have been compared with those of other methods.

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EXPERIMENTAL

Enzyme preparation

Rats (male Wistar) were killed by cervical dislocation and the brains rapidly removed and cleaned of adhering dura and blood vessels on ice.

Insect nervous tissues were obtained from adult male and female cockroaches (*Periplaneta americana*) maintained on Purina Dog Chow® and water *ad libitum*. Tissues were dissected from cold anaesthetised animals and placed in 1.5-ml polypropylene microtubes on ice.

Nervous tissues were homogenised in three volumes (rat) or ten volumes (cockroach) of ice-cold 50 m*M* Tris–HCl buffer (pH 7.4) containing 1 m*M* dithiothreitol (DTT) and centrifuged at 20 000 *g* for 20 min. The pellet was discarded. The supernatant was combined with ammonium sulphate to 60% saturation (solid ammonium sulphate was used for rat brain preparations and saturated ammonium sulphate in water for cockroach nervous tissue preparations) and then centrifuged at 20 000 *g* for 15 min. The supernatant was discarded. After resuspension in approximately ten volumes of 50 m*M* Tris–HCl buffer (pH 7.4) containing 1 m*M* dithiothreitol, the sample was frozen (−20°C) until analysed.

Enzyme assay

Typical incubation mixtures contained 50 m*M* Tris–HCl (pH 7.4); 0.25 m*M* 6,7-dimethyl-5,6,7,8-tetrahydropterine ($DMPH_4$); 0.5 m*M* DTT; 0.6 m*M* NADP; 0.25 *M* glucose; 0.065 units/ml glucose dehydrogenase (calf liver); 1 m*M* L-tryptophan and 10 μl enzyme preparation in a total volume of 100 μl.

Incubations were maintained for 40 min at 37°C with gentle agitation in 1.5-ml polypropylene microtubes until stopped by the addition of an equal volume of 0.1 *M* perchloric acid containing 0.1 m*M* sodium metabisulphite and 0.25 m*M* EDTA. The mixture was centrifuged (28 000 *g*) in an Eppendorf 5412 bench top centrifuge for 10 min and the supernatant injected directly onto the chromatographic column.

Chromatography

Separation and quantitation was achieved by reversed-phase HPLC with coulometric electrochemical detection modified from Sloley and Downer[11]. Samples (1–5 μl) were introduced onto the column by means of a WISP 710B (Waters, Mississauga, Canada) autosampler. The column (Beckman Ultrasphere RP-18, 5 μm particle size, 25 cm × 4.6 mm I.D.) was protected by a guard column (Brownlee RP-18, 7 μm particle size, 15 × 3.2 mm I.D., Brownlee Lab., Santa Clara, CA, U.S.A.). The mobile phase (0.083 *M* sodium acetate, 0.033 *M* citric acid, 0.1 m*M* EDTA, 10% methanol, pH 4.7) was pumped through the column by means of a Waters M45 pump at a flow-rate of 0.8 ml/min. Water used in the preparation of all solutions was 18.3 MΩ deionised water obtained from a Barnstead Nasnopure II deionising system and passed through a disposable reversed-phase column (Baker-10 SPE, 3 ml, J. T. Baker, Phillipsburg, NJ, U.S.A.). The aqueous portion of the mobile phase was filtered through a 22 μm pore filter and degassed by vacuum.

Eluted compounds were detected electrochemically using a coulometric detection systemn (ESA 5100A, Bedford, MA, U.S.A.) with two electrodes set at oxidising

potentials of 0.43 and 0.65 V, respectively. A guard cell located before the injection valve set at 0.95 V pre-oxidised all potential contaminants in the mobile phase before they could register at the detector electrodes.

5-HTP, 5-HT, 5-HIAA and NA-5-HT were measured at the first electrode and TRP and $DMPH_4$ were measured at the second electrode although 5-HTP and $DMPH_4$ could be oxidised at both electrodes. Identification of the compounds was by co-chromatography with standards and by the specific oxidation characteristics of the various compounds.

Protein was measured by the method of Lowry *et al.*[14] using bovine serum albumin as standard.

All chemicals with the exception of methanol (HPLC grade, Caledon) were obtained from Sigma (St. Louis, MO, U.S.A.).

RESULTS AND DISCUSSION

Chromatography

Previous reports[11,15] demonstrated baseline separation of TRP, 5-HTP, 5-HT, 5-HIAA and NA-5-HT. The mobile phases used in these studies, however, do not separate 5-HTP and 5-HT from the cofactor $DMPH_4$ which must be present in the incubation mixture. In our system we obtained these separations by reducing the molarity and methanol content of the mobile phase.

Fig. 1 shows typical chromatograms of standards and enzyme incubations. The formation of 5-HTP in the enzyme reaction is clearly demonstrated; it was not present or formed by acid-denatured enzyme preparations. In addition, no detectable

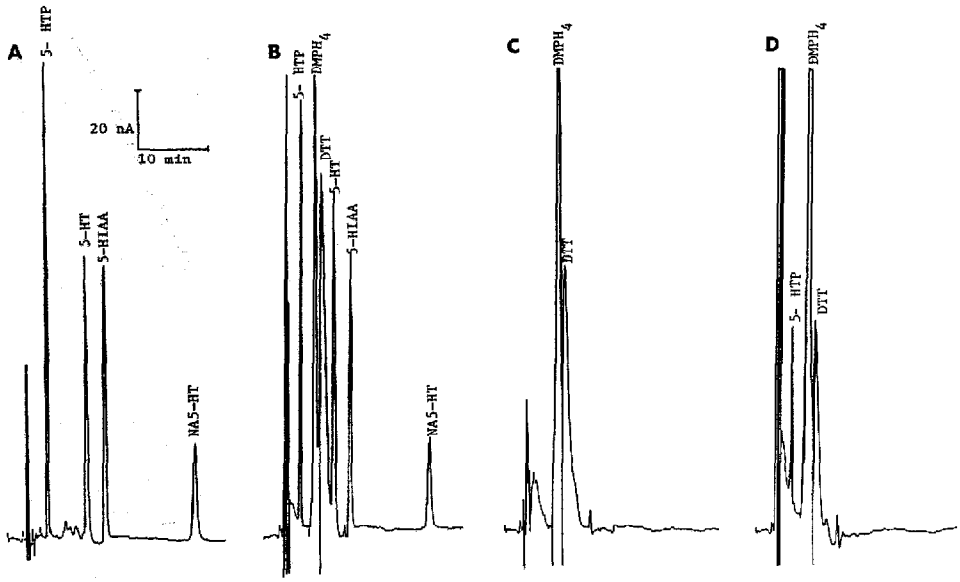

Fig. 1. Chromatograms of 1 ng standards (A), perchloric acid extract of enzyme reaction mixture with 900 pg of each standard without incubation (B), extract of 30-min incubation with no enzyme preparation (C) and extract of 30-min incubation with enzyme preparation (D).

amounts of 5-HT, 5-HIAA or NA-5-HT were produced during incubation. An improved resolution of 5-HTP and 5-HT from $DMPH_4$ can be accomplished by reducing the amount of methanol in the mobile phase (from 10% to 8%) although this improvement is at the expense of speed since the time for HPLC analysis is increased from 25 to 40 min.

The lower limit of detection for 5-HTP was 10 pg per 5 μl injection, which is equivalent to a lower limit of 0.6 pmole/mg protein min for the assay.

Validity of the assay

The production of 5-HTP by the enzyme is linear both with respect to time (for at least 40 min) and protein concentrations (Figs. 2 and 3) and is absolutely dependent on the presence of TRP and $DMPH_4$ in the incubation (Table I). The omission of glucose, NADP and glucose dehydrogenase greatly reduced the amount of 5-HTP formed. The omission of DTT caused a slight increase in the amount of 5-HTP produced. These results are consistent with those that assessed rabbit hindbrain tryptophan hydroxylase[16]. Pargyline (1.0 m*M*) slightly inhibited the reaction (Table I) and caused no detectable accumulation of 5-HT.

The tryptophan hydroxylase activity in crude enzyme preparations of rat brain, cockroach cerebral ganglia, suboesophageal ganglia and thoracic ganglia is presented in Table II. Tryptophan hydroxylase activity was detected in all these tissues. The

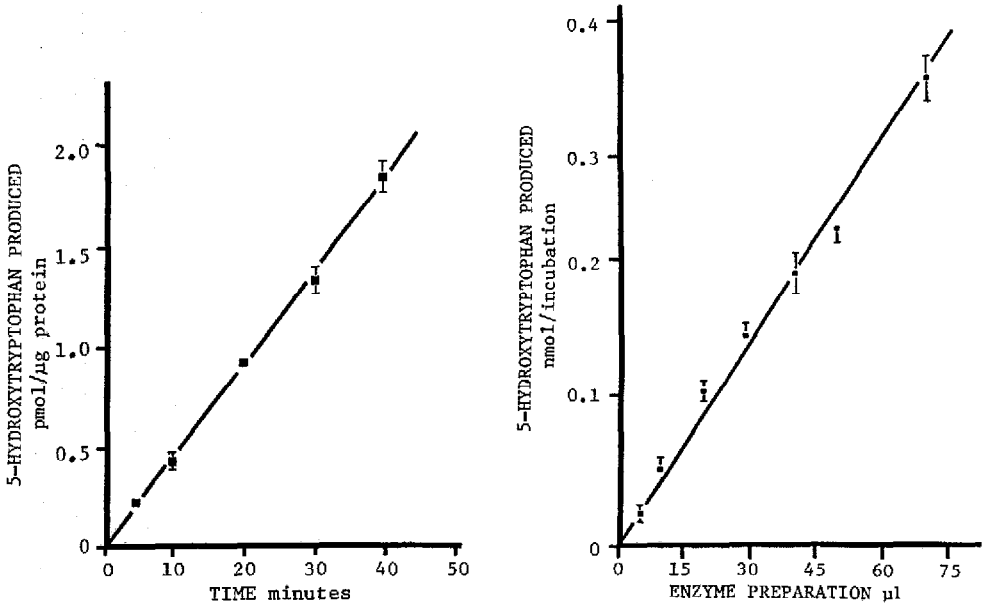

Fig. 2. Time course of production of 5-hydroxytryptophan in typical incubations of crude rat brain enzyme preparations. Conditions are as described in enzyme assay section. Values are mean ± S.E.M. (standard error of the mean) based on three determinations.

Fig. 3. Effect of amount of enzyme preparation on production of 5-hydroxytryptophan. Conditions are as described in enzyme assay section except total volume is increased to 400 μl. The concentration of protein in the rat brain enzyme preparation was 12.2 mg/ml. Values are mean ± S.E.M. based on three determinations.

TABLE I

PROPERTIES OF RAT BRAIN TRYPTOPHAN HYDROXYLASE

Values are mean ± S.E.M. based on four determinations.

Asssay conditions	*5-HT produced (%)*
Standard incubation*	100 ± 4.0
Omit $DMPH_4$	0
Omit TRP	0
Omit DTT	112 ± 1.1
Omit glucose	104 ± 4.0
Omit NADP	99 ± 2.7
Omit glucose dehydrogenase	43 ± 1.4
Omit NADP, glucose and glucose dehydrogenase	27 ± 1.4
Include pargyline (1.0 m*M*)	87 ± 1.2
Omit tryptophan hydroxylase enzyme preparation	0
Control stopped at time = 0 min with perchloric acid	0

* 50 m*M* Tris–HCl; 1 m*M* TP; 0.25 m*M* $DMPH_4$; 0.5 m*M* DTT; 0.6 m*M* NADP, 0.25 *M* glucose; 0.065 units/ml glucose dehydrogenase.

rat brain tryptophan hydroxylase activity estimated by this procedure was similar to values obtained using fluorometric and radiometric methods[4,5,16–18]. Tryptophan hydroxylase activity in crude homogenates of cockroach cerebral ganglia has been estimated at 0.34 nm/h · g wet tissue[19], which is considerably lower than its level in mammalian preparations. This is also reflected in our results (Table II).

In conclusion, a HPLC procedure coupled with electrochemical detection has provided a simple, specific and highly sensitive method for the measurement of tryptophan hydroxylase as isolated from different tissues.

TABLE II

SPECIFIC ACTIVITY OF TRYPTOPHAN HYDROXYLASE IN CRUDE ENZYME PREPARATIONS OF RAT AND COCKROACH NERVOUS TISSUES

Values are mean ± S.E.M. based on four determinations.

Tissue source	*Specific activity (nmol/mg protein · min)*	*Ref.*
Rat brain ammonium sulphate precipitate	0.054 ± 0.002	Present study
Rat brain crude extract	0.103	14
Pig brainstem crude extract	0.013	1
Pig brainstem ammonium sulphate precipitate	0.025	1
Rabbit hindbrain crude extract	0.036	2
Rabbit brain crude extract	0.068	16
Cockroach cerebral ganglia ammonium sulphate precipitate	0.0013 ± 0.0001	Present study
Cockroach suboesophageal ganglia ammonium sulphate precipitate	0.0014 ± 0.0001	Present study
Cockroach thoracic ganglia ammonium sulphate precipitate	0.0005 ± 0.00002	Present study

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