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A SENSITIVE RADIOMETRIC ASSAY FOR TRYPTOPHAN-5-HYDROXYLASE

ROLF HÅKANSON and GARY J. HOFFMAN

Department of Pharmacology, University of Lund, Lund, Sweden

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Abstract—A simple and sensitive radiometric micromethod for the study of tryptophan-5-hydroxylase is described. ^{14}C-5-hydroxytryptophan (5-HTP), which is the product of the reaction, is separated from the ^{14}C-tryptophan substrate by thin-layer chromatography and assayed by liquid scintillation counting.

The enzyme activity in some tissues of the rat was measured. The pineal gland, which is known to store large amounts of 5-hydroxytryptamine, had the highest activity of all tissues studied. Stomach wall and duodenum had only moderate activities, whereas rat liver was a rich source of a 5-HTP-producing enzyme, possibly identical with non-specific phenylalanine hydroxylase. The enzyme activity of all tissues studied was considerably reduced following homogenization, suggesting that the formation of 5-HTP requires some co-factor, which occurs in high concentration intracellularly. Tryptophan hydroxylase extracted from rat pineals could be reactivated by the addition of a tetrahydropteridine derivative, but only in the presence of 2-merkaptoethanol.

TRYPTOPHAN-5-hydroxylase is a key enzyme in the biosynthesis of 5-hydroxytryptamine.[1–3] The enzyme, which catalyses the conversion of tryptophan to 5-hydroxytryptophan (5-HTP), is conventionally assayed by fluorometric[4–6] and radioisotopic methods.[7–9] This report describes a sensitive and specific radiometric assay for tryptophan-5-hydroxylase which for its simplicity has distinct advantages over previously described techniques. The method has been used to determine the enzyme activity in some tissues of the rat.

MATERIAL AND METHODS

All preliminary experiments were performed using the intact rat pineal gland as enzyme source. The pineal of the adult rat is fairly uniform in size, weighing approximately 1 mg. The glands were incubated with 4 μg ^{14}C-DL-tryptophan (8 mc/mM; Radiochemical Centre, Amersham) or ^{14}C-L-tryptophan (30 mc/mM) in rat Ringer solution; the total volume of the incubation mixture was 60 μl. If not otherwise stated *m*-hydroxybenzylhydrazine (NSD-1015; Ferrosan, Sweden)—a potent DOPA decarboxylase inhibitor[10, 11]—was added to a final concentration of 10^{-5} M in order to protect newly formed 5-HTP from destruction by pineal DOPA decarboxylase.[11] The reaction was carried out under 95% oxygen + 5% carbon dioxide at 37° in a metabolic shaker and was stopped by the addition of 1 ml acetone–hydrochloric acid (0·1 N) (95:5), containing 200 μg each of non-radioactive 5-HTP and tryptophan. After homogenization, the precipitate formed was spun down and 0·35 ml of the

supernatant was transferred to a Thunberg tube and evaporated to dryness under reduced pressure. The dry material was taken up in a few drops of the acetone–hydrochloric acid mixture and applied to a silica gel (Kieselgel G, Merck) thin-layer-chromatoplate (500 μ thick). 5-HTP could be quantitatively separated from tryptophan in less than 1 hr with a solvent system of 14% NaCl in water. The plates were developed for about 20 cm, and then sprayed with 0·2% ninhydrin in acetone while still slightly damp. The spots containing 5-HTP (R_f value 0·90) and tryptophan (R_f value 0·75) were scraped off and transferred to counting vials with 0·2 ml water added as eluant. The radioactivity was measured by liquid scintillation after the addition of 10 ml dioxane–phosphor mixture.[9] Under these conditions the quenching was fairly constant and less than 5 per cent; the results presented are calculated from uncorrected values. Enzyme activity is expressed as per cent of tryptophan converted to 5-HTP. Recovery of ^{14}C-5-HTP and ^{14}C-tryptophan added to incubation mixtures was 80–90 per cent. The ^{14}C-5-HTP-containing silica gel material had very little contamination from the ^{14}C-tryptophan substrate. A blank value was obtained using boiled tissue samples. All values were corrected for this heated enzyme blank, which never exceeded 0·03 per cent of substrate radioactivity.

RESULTS AND DISCUSSION

The reaction velocity was rectilinear with time for at least 3 hr and proportional to the amount of pineal tissue added (Fig. 1). With very high tissue concentrations, however, the reaction velocity was not always linearly related to the amount of enzyme.

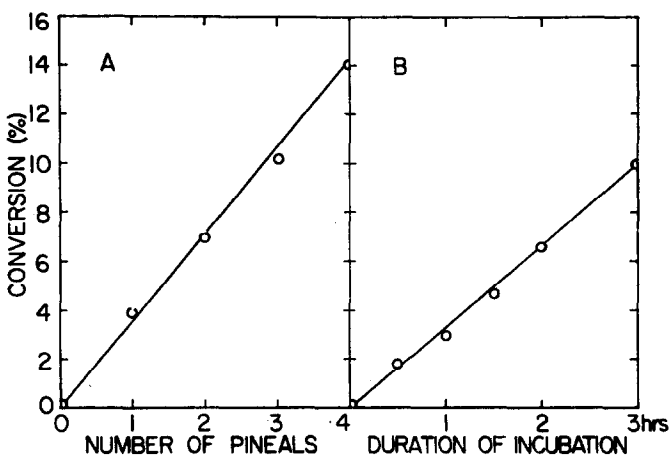

FIG. 1. The relation between enzyme activity and amount of tissue added (A), and between 5-hydroxytryptophan formation and duration of incubation (B). In A the reaction time was 2 hr; in B each sample contained 2 intact pineals. The substrate was ^{14}C-DL-tryptophan (sp. act. 8 mc/mM) NSD-1015 was added to the incubation mixture (see Methods).

Addition of a decarboxylase inhibitor (NSD-1015) increased the measured enzyme activity more than two-fold, presumably by interfering with the further metabolism of the product.

The reaction product was identified as ^{14}C-5-HTP by thin-layer chromatography with authentic 5-HTP in solvent systems of 14% NaCl in water (Fig. 2), *n*-butanol–

pyridine–acetic acid–water (15:2:3:5) and ethanol–diethyl ether–ammonia–water (10:10:1:4).

Tryptophan hydroxylase activity was estimated in a variety of rat tissues (Table 1). The data in Table 1 refer to non-homogenized tissue samples; homogenization caused a severe reduction in the enzyme activity of all tissues studied. The pineal gland, which contains extremely high amounts of 5-hydroxyindoles,[2, 3] had the highest tryptophan hydroxylase activity. Normal rat mast cells store 5-hydroxytryptamine in appreciable amounts,[2, 3] but peritoneal saline washings, which contain large quantities of mast cells

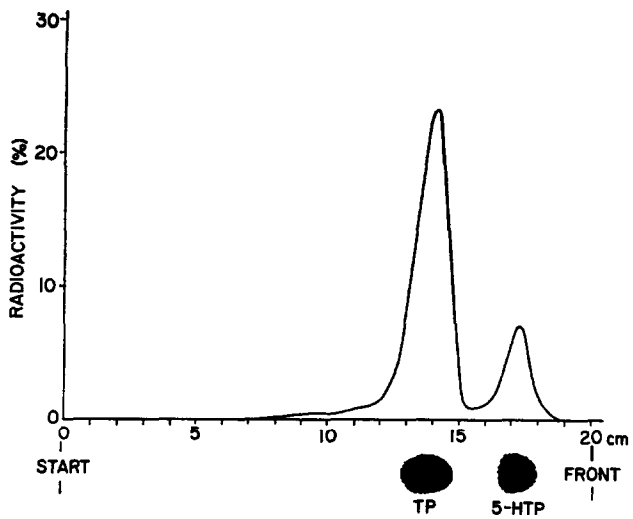

FIG. 2. Radioactivity profile of a thin-layer chromatogram: Extract obtained from 4 intact pineals incubated with ^{14}C-DL-tryptophan for 3 hr. Solvent system: 14% NaCl. Fractions of 4 mm were collected and counted by liquid scintillation. The positions of the reference compounds are shown by photography after ninhydrin treatment. 5-HTP, 5-hydroxytryptophan; TP, tryptophan.

TABLE 1. TRYPTOPHAN HYDROXYLASE ACTIVITY IN VARIOUS TISSUES OF THE RAT

Organ	Tryptophan hydroxylase activity*	
Liver	1·45 ± 0·35	(4)
Stomach wall	0·032 ± 0·009	(4)
Duodenum	0·009 ± 0·002	(4)
Jejunum	0·006 ± 0·003	(4)
Pineal gland	3·05 ± 0·25	(20)
Cerebral cortex	0·002 ± 0·001	(4)
Mast cells†	0	(4)

* Enzyme activity is expressed as per cent of tryptophan hydroxylated per mg tissue in 1 hr. Mean ± S.E.M. (number of experiments).
All incubations were performed with ^{14}C-L-tryptophan as substrate. The weight of the tissue sample was usually 5–10 mg.

† Mast cells were collected by intraperitoneal saline washings as described by Schayer.[12] A considerable portion of this material consisted of lymphocytes and leukocytes.

had no enzyme activity (see also Ref. 4, 13). Only moderate enzyme activity was observed in the stomach wall and in the duodenum, whereas the rat liver was a rich source of a 5-HTP-producing enzyme, possibly identical with non-specific phenylalanine hydroxylase.[14] The enzyme activity of all tissues studied was considerably reduced (to approx. 20 per cent of normal) by intraperitoneal injection of 400 mg/kg of α-propyldopacetamide (Hässle, Sweden), a potent inhibitor of tryptophan hydroxylase.[6, 15, 16] The tissue samples were removed 1 hr after injection of the inhibitor.

The reduction of enzyme activity following homogenization may suggest that the formation of 5-HTP depends on some co-factor which occurs in high concentration intracellularly. Pineal extracts were usually prepared by homogenizing 10 mg tissue in 0·5 ml rat Ringer solution containing 10^{-5} M NSD–1015. In the presence of 2-merkaptoethanol (5×10^{-2} M) and ferrous ion (10^{-4} M) the pineal enzyme was reactivated by a tetrahydropteridine derivative ($DMPH_4$; 10^{-3} M, $K_m \sim 10^{-5}$), which is also a co-factor of rat liver tryptophan hydroxylase;[15] the procedure adopted was the one described for the reactivation of tryptophan hydroxylase from a murine mastocytoma.[17] In the absence of 2-merkaptoethanol, $DMPH_4$ failed to reactivate the enzyme. NADH, NADPH and biopterin (Sigma) added separately or together in various concentrations from 10^{-6} M to 10^{-4} M had no effect (with or without 2-merkaptoethanol) and the addition of ascorbic acid[7, 18] or dehydroascorbic acid (10^{-5}–10^{-3} M)[19] resulted in an enzyme activity which was only a fraction of that observed with intact pineals.

It is possible that the pineal enzyme is identical with the tryptophan hydroxylase previously demonstrated in the brainstem.[9, 18, 20, 21]

Note added in proof.—After this manuscript had been submitted for publication, Lovenberg, Jecquier and Sjoerdsma (*Science* **155**, 217 (1967)) reported high tryptophan hydroxylase activity in the pineal gland and some other mammalian tissues.

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