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FURTHER STUDIES ON TRYPTOPHAN HYDROXYLASE IN RAT BRAINSTEM AND BEEF PINEAL

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Abstract—Soluble tryptophan hydroxylating enzymes have been obtained from both the beef pineal gland and rat brainstem. The enzymes have a pH optimum of 7·5 and appear to be typical aromatic ring hydroxylases. Although an iron requirement of the enzymes can be demonstrated by iron chelators, exogenous iron is not absolutely required for activity. Tetrahydrobiopterin was more effective than 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8 tetrahydropteridine as a cofactor for the enzymes. The beef pineal enzyme hydroxylates phenylalanine at approximately the same rate as it does tryptophan, whereas the brain enzyme showed no detectable activity toward phenylalanine. Finally, catechol compounds are effective inhibitors of the enzymes, presumably because of their ability to chelate iron.

TRYPTOPHAN hydroxylase is the rate-limiting enzymic step in serotonin biosynthesis. The first mammalian tissue found to exhibit tryptophan hydroxylase activity *in vitro* was the liver.[1] It subsequently was shown, however, that this activity arose from the nonspecificity of phenylalanine hydroxylase and several lines of evidence suggested that this enzyme did not participate substantially in normal biosynthesis of serotonin.[2] More recently, several authors have reported the presence of tryptophan hydroxylase activity in brainstem and carcinoid tumor.[3–7] Work from this laboratory[3,8] has established that malignant mouse mast cell tumors, pineal gland and brainstem are active sources of tryptophan hydroxylase. Examination of crude extracts of these tissues indicates that tryptophan hydroxylase is a typical mixed function oxidase with properties very similar to other aromatic amino acid ring hydroxylases.[9]

The present communication describes the preparation and characterization of a soluble tryptophan hydroxylase from rat brainstem and beef pineal gland. The enzyme is not significantly inhibited by numerous tryptophan analogues but is strongly inhibited by catechol compounds.

MATERIALS AND METHODS

Materials. L-tryptophan-3-^{14}C (50 μc/μmole) was obtained from Nuclear Chicago

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‡ The abbreviations used in this paper are: $DMPH_4$, 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8 tetrahydropteridine; 5-HTP, 5-hydroxytryptophan; and H-2254, 2-propyl-3,4 dihydroxyphenylacetamide. The latter compound was generously supplied by Dr. Hans Corrodi, of A.B. Hassel, Gotenborg, Sweden.

Corp. 2-Amino-4-hydroxy-6,7-dimethyl, 5,6,7,8 tetrahydropteridine ($DMPH_4$)‡ was obtained from Aldrich Chemical Co. A sample of 2-amino-4-hydroxy-6-dihydroxypropyltetrahydropteridine (tetrahydrobiopterin) was prepared chemically by Dr. Ross Shiman, National Institute of Mental Health, Bethesda, Md. Other materials used were obtained from commercial sources. Rats were male Sprague–Dawley weighing 170–230 g.

Enzyme preparation and assay. Rat brainstem was removed immediately after decapitation; beef pineal glands were obtained at a slaughter house within 10 min of exsanguination. All tissues were chilled immediately. The enzymes used for most studies were prepared through the ammonium sulfate fractionation step (see below).

The hydroxylation of tryptophan to 5-hydroxytryptophan (5-HTP) was measured using a typical assay as follows: 0·2 ml of the partially purified enzyme incubated in the presence of 0·25 M Tris-acetate buffer (pH 7·5), 0·7 mM L-5-HTP, 0·4 mM pargyline hydrochloride, 1 mM $DMPH_4$, 0·05 M 2-mercaptoethanol, 0·1 mM ferrous ammonium sulfate, and 0·12 mM L-tryptophan-2-^{14}C (16·7 μc/μmole) in a total volume of 0·3 ml. The 5-HTP formed in this reaction was determined by the radioassay developed in this laboratory.[9] The hydroxylation of phenylalanine was measured by the method of Guroff and Abramowitz.[10] Protein was determined by a modification of the phenol reagent protein assay.[11]

RESULTS

Purification of beef pineal gland and rat brainstem tryptophan hydroxylase. Tryptophan hydroxylase can be purified about 5-fold from beef pineals by ammonium sulfate fractionation. As shown in Table 1, dialysis of the 30,000 *g* supernatant fraction of an homogenate against 2-mercaptoethanol solutions resulted in a 2- to

TABLE 1. PURIFICATION OF TRYPTOPHAN HYDROXYLASE FROM BEEF PINEAL*

Enzyme preparation	Total activity (mμmoles/hr)	Protein (mg)	Enzyme specific activity (mμmoles/mg prot./hr)
Homogenate not dialyzed	74·5	168	0·44
Homogenate dialyzed	178	162	1·10
Supernatant 30,000 *g* not dialyzed	81	88	0·92
Supernatant 30,000 *g* dialyzed	159	80	2·0
Precipitate 30,000 *g* not dialyzed	15·1	80	0·19
Precipitate 30,000 *g* dialyzed	44·5	74	0·60
Supernatant 30,000 *g* 0–30% $(NH_4)_2SO_4$ dialyzed	19·3	20·6	0·94
Supernatant 30,000 *g* 30–40% $(NH_4)_2SO_4$ dialyzed	6·5	8·5	0·77
Supernatant 30,000 *g* 40–50% $(NH_4)_2SO_4$ dialyzed	3·0	8·5	0·35
Supernatant 30,000 *g* 50–60% $(NH_4)_2SO_4$ dialyzed	112·5	20·0	5·60

* Homogenate (1:5) of ten beef pineals (2 g of tissue) with 0·05 M Tris buffer, pH 7·4, was carried out with a glass motor-driven homogenizer. Supernatant fraction was obtained by centrifuging at 30,000 *g* for 20 min. The precipitate was suspended in 5 ml 0·05 M Tris, pH 7·4. Homogenate, supernatant fraction and precipitate were dialyzed overnight against 0·1 M 2-mercaptoethanol in 0·05 M Tris, pH 7·4. The dialyzed supernatant was subjected to ammonium sulfate precipitation; the fractions obtained were centrifuged at 10,000 *g* for 10 min, the precipitate suspended in 2 ml 0·05 M Tris, pH 7·4, and dialyzed 4 hr against 0·1 M 2-mercaptoethanol in 0·05 M Tris, pH 7·4.

3-fold stimulation of enzyme activity. The enzyme precipitated from this dialyzed solution between 50 and 60 per cent saturation with ammonium sulfate. This enzyme preparation was a convenient material for further study of the enzyme properties.

Brainstem tryptophan hydroxylase can be prepared in a similar manner (Table 2). The intracellular distribution and the phenomenon of marked stimulation of this enzyme upon dialysis against 2-mercaptoethanol have been reported previously.[12] Since this enzyme precipitated over a broader range of ammonium sulfate concentration than the pineal enzyme, for most studies the brainstem protein precipitating between 30 and 60 per cent ammonium sulfate saturation was used. The enzyme could be further purified by adsorption and elution from calcium phosphate gel and refractionation with ammonium sulfate as indicated in Table 2.

TABLE 2. PURIFICATION OF TRYPTOPHAN HYDROXYLASE FROM RAT BRAINSTEM*

Enzyme preparation	Total activity (mμmoles/hr)	Protein (mg)	Enzyme specific activity (mμmoles/mg prot./hr)
Supernatant 30,000 *g* dialyzed	90	165	0·54
Supernatant 30,000 *g* 30–60% $(NH_4)_2SO_4$ dialyzed	46	47	0·97
$CaPO_4$ gel eluate dialyzed	27	25·2	1·07
$CaPO_4$ gel eluate 0–40% $(NH_4)_2SO_4$ dialyzed	25·4	13·6	1·87

* Seventeen rat brainstems (9 g of tissue) were homogenized with a glass motor-driven homogenizer in 0·05 M Tris Cl buffer, pH 7·5 (1:3). After centrifugation at 30,000 *g* for 15 min, the supernatant was dialyzed overnight against 0·1 M 2-mercaptoethanol with 0·05 M Tris, pH 7·5. The 30,000 *g* supernatant was precipitated with ammonium sulfate and the 30–60 per cent fraction resuspended in 10 ml of 0·05 M Na phosphate buffer, pH 6·0, and 0·1 M 2-mercaptoethanol and dialyzed in 200 vol. of the same solution for 4 hr. One mg of $CaPO_4$ gel per 2 mg protein was next added to the enzyme solution and after adsorption for 15 min at 0° the suspension was centrifuged at 10,000 *g* for 10 min. The calcium phosphate precipitate was eluted with 1 ml of 0·5 M sodium phosphate buffer, pH 7·, containing 0·1 M 2-mercaptoethanol and centrifuged at 10,000 *g* for 10 min. This was repeated twice more. An ammonium sulfate precipitation was carried out on the combined eluates and the 0–40 per cent fraction was resuspended in 1 ml of 0·05 M Tris Cl buffer, pH 7·5, and dialyzed overnight in 0·05 M Tris Cl buffer, pH 7·5, and 0·1 M 2-mercaptoethanol.

Properties and requirements of the enzymes prepared by ammonium sulfate fractionation. The requirements for assay of rat brainstem and beef pineal enzymes *in vitro* are the same as those reported for the crude tissue homogenates.[3,9] These enzymes showed a complete dependence on the reduced pteridine cofactor ($DMPH_4$ or tetrahydrobiopterin) and molecular oxygen and were strongly stimulated by 2-mercaptoethanol. Ferrous iron stimulated the activity of the pineal enzyme about 2-fold (Fig. 1), but failed to activate the brainstem enzyme. The calculated iron K_m value for iron with the pineal enzyme is about $2{\cdot}5 \times 10^{-5}$ M.

The iron chelators α,α-dipyridyl (10^{-4} M) and *o*-phenanthroline (10^{-4} M) completely inhibit the activity of both enzymes suggesting that iron is a necessary component of the enzyme system. This inhibition can be completely reversed by the addition of ferrous iron and, in fact, the activity was usually higher than the same enzyme which had not been pretreated with an iron chelator (Table 3). Addition of iron alone (not shown in this table) to this type of enzyme preparation usually results in a 2-fold stimulation of activity.

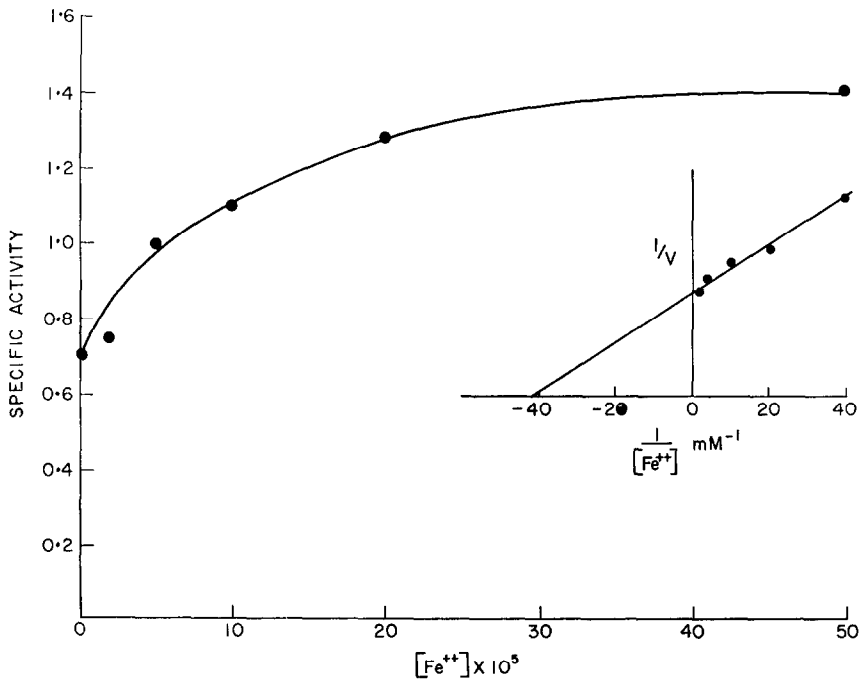

FIG. 1. Effect of ferrous ion addition on the activity of pineal tryptophan hydroxylase.

TABLE 3. SUPPRESSION OF THE ENZYME INHIBITION DUE TO α α'-DIPYRIDYL OR *o*-PHENANTHROLINE BY Fe^{++}*

	Enzyme specific activity (μmoles/mg protein)
Control (without Fe^{++} added)	0·59
+ 10^{-4} M α α'-dipyridyl	0·01
+ 10^{-4} M α α'-dipyridyl + 10^{-4} M Fe^{++}	1·85
+ 10^{-4} M *o*-phenanthroline	0·01
+ 10^{-4} M *o*-phenanthroline + 10^{-4} M Fe^{++}	1·88

* The beef pineal enzyme was prepared as described under Methods. The 40–60 per cent $(NH_4)_2SO_4$ fraction was used for the assay; each tube contained 0·72 mg of protein.

The pH optimum for tryptophan hydroxylation by the brainstem and beef pineal enzymes was 7·5. The effect of pH on the reaction catalyzed by the brainstem enzyme is shown in Fig. 2.

The effect of substrate and cofactor concentration on reaction rates was measured and the K_m values determined from double reciprocal plots. The typical assay system was used in these experiments with incubation periods of 60 min. The rate of hydroxylation appeared to be constant during this time period.[9] Both the pineal and brainstem enzymes were similar in this regard, exhibiting K_m values of about 3×10^{-4} M for tryptophan and about 3×10^{-5} M for $DMPH_4$. As seen in Table 4, the use of

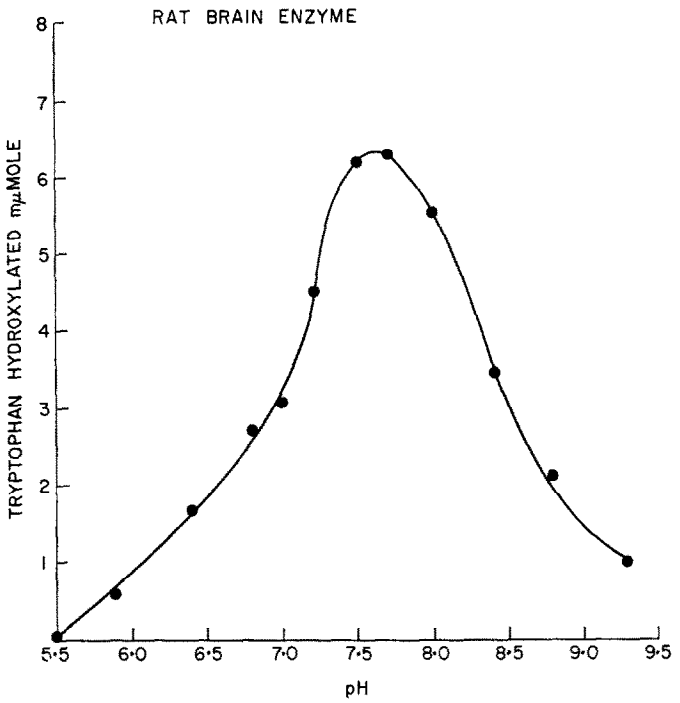

FIG. 2. Effect of pH on tryptophan hydroxylated by the rat brainstem enzyme. Reaction mixtures made with 0·25 M Tris-acetate buffer adjusted to the pH values indicated were used.

TABLE 4. EFFECTIVENESS OF $DMPH_4$ AND TETRAHYDROBIOPTERIN AS COFACTORS FOR RAT BRAINSTEM TRYPTOPHAN HYDROXYLASE*

Cofactor	Enzyme specific activity (mμmoles/mg prot./hr)	K_m†
None	0·07	
$DMPH_4$ 10^{-3} M	1·60	3×10^{-5} M
Tetrahydrobiopterin 3×10^{-5} M	2·70	5×10^{-6} M

* The enzyme was prepared as described under Methods. The 30–60 per cent $(NH_4)_2SO_4$ fraction of the 30,000 *g* supernatant was used in the assay; the enzyme preparation was dialyzed for 4 hr against 0·1 M 2-mercaptoethanol before the assay; 3·6 mg of protein was used for each assay.

† The K_m values for $DMPH_4$ and tetrahydrobiopterin were computed from double reciprocal plots.

tetrahydrobiopterin rather than $DMPH_4$ results in a higher rate of hydroxylation. The approximate K_m value for tetrahydrobiopterin was also appreciably lower than that seen with $DMPH_4$.

Bovine serum albumin was found to stimulate the tryptophan hydroxylating activity of the crude supernatant of brainstem, as shown in Fig. 3. Bovine serum albumin also stimulated to a similar degree the enzymatic activity of the 30–60 per cent ammonium sulfate fraction of the crude supernatant. However, no enhancement of the more purified calcium phosphate gel eluate was noted.

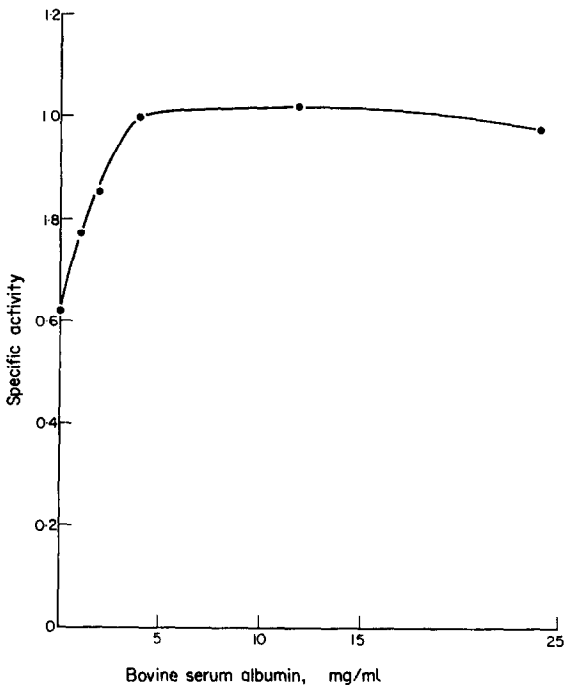

FIG. 3. Effect of bovine serum albumin addition on the tryptophan hydroxylating activity of rat brainstem. The crude supernatant fraction was dialyzed overnight against 0·1 M 2-mercaptoethanol in 0·05 M Tris-acetate buffer, pH 7·5, and assayed as described under Methods.

Specificity. Since L-phenylalanine is a substrate for the tryptophan hydroxylating enzyme of malignant mouse mast cells[8] and a competitive inhibitor of tryptophan hydroxylation in brainstem and pineal enzyme preparations,[9] attempts were carried out to show phenylalanine hydroxylase activity with these tissue preparations. It was found that the pineal enzyme can hydroxylate both phenylalanine and tryptophan at equivalent rates, whereas the brainstem enzyme appears to be specific for tryptophan (Table 5). The ratio of phenylalanine hydroxylase to tryptophan hydroxylase activity was about 1:1 for the beef pineals and less than 1:100 for the rat brainstem. The phenylalanine hydroxylating activity of the pineal exhibited the same requirements *in vitro* as the tryptophan hydroxylase requirements for this tissue (Table 5).

Inhibitors. The assay of tryptophan hydroxylase is done in the presence of L-5-HTP and initially it was felt that there was little or no product inhibition of the reaction. Using the partially purified enzyme from brainstem and examining the reaction over wider range L-5-HTP concentrations, however, it is apparent that product inhibition does occur (Fig. 4). Tryptophan hydroxylase assays are normally done using levels of trapping L-5-HTP which result in inhibition by less than 20 per cent of maximal rates. The extremely high levels of product required for inhibition indicate that this is probably not a physiological control mechanism for serotonin synthesis. Analogous experiments were done using serotonin and D-5-HTP as inhibitors. Concentrations of up to 10^{-4} M for serotonin and 2×10^{-3} M for D-5-HTP caused no observable inhibition.

TABLE 5. COMPARISON BETWEEN THE TRYPTOPHAN HYDROXYLATING AND PHENYLALANINE HYDROXYLATING ACTIVITY IN BEEF PINEAL GLANDS AND RAT BRAINSTEM*

Enzyme	Tryptophan hydroxylated (mμmoles/mg prot.)	Phenylalanine hydroxylated (mμmoles/mg prot.)
Beef pineal: Complete system	3·8	3·0
$-Fe^{++}$	2·4	2·9
$-DMPH_4$	0·17	<0·01
$-O_2$	0·25	0·15
Rat brainstem: Complete system	1·18	<0·01
$-Fe^{++}$	1·17	<0·01
$-DMPH_4$	0·07	<0·01
$-O_2$	0·01	<0·01

* Tissues were homogenized with a glass motor-driven homogenizer in 2 vols. of 0·05 M Tris buffer, pH 7·4. The homogenate was centrifuged at 30,000 *g* for 10 min, the supernatant dialyzed overnight against 0·1 M 2-mercaptoethanol in 0·05 M Tris, pH 7·4; 0·2 ml of the dialyzed supernatant was used in the assay. The complete system consisted of 0·2–0·4 ml of the partially purified enzyme incubated in the presence of 0·25 M Tris-acetate buffer (pH 7·5), 1 mM $DMPH_4$, 0·05 M 2-mercaptoethanol and 0·1 mM ferrous ammonium sulfate in a total vol. of 0·3–0·5 ml.

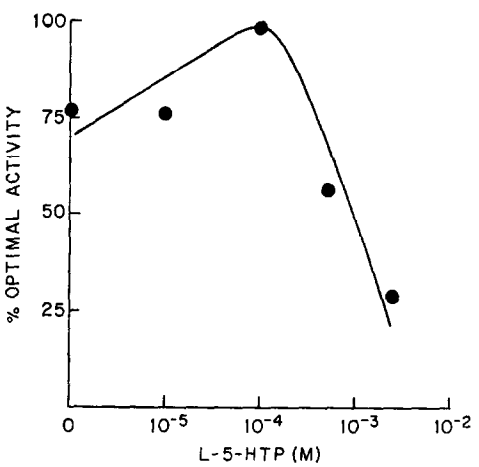

FIG. 4. Effect of L-5-HTP on tryptophan hydroxylated by the partially purified rat brainstem enzyme. Each point represents the average of two experiments done in duplicate.

The finding that catechol compounds are potent inhibitors of the mast cell tryptophan hydroxylase[8] and brain tyrosine hydroxylase[13] led to the study of these compounds as inhibitors of brainstem and pineal tryptophan hydroxylase. As seen in Table 6, all the catechol compounds tested exerted strong inhibition, the most potent catechol inhibitor being H-2254. The effect of cofactor concentrations on the reaction rates in the presence and absence of the inhibitor is shown in Fig. 5. The catechol compounds appear to be typically noncompetitive inhibitors with respect to the cofactor. Similar experiments with the beef pineal enzyme had shown that norepinephrine is a noncompetitive inhibitor with respect to either substrate or cofactor.[9] The experiments reported here were done in the absence of exogenous iron since it was found that ferrous ion could reverse the inhibition. It, therefore, appears that catechols may inhibit the enzyme in a manner analogous to the iron chelators α,α-dipyridyl and *o*-phenanthroline.

TABLE 6. INHIBITORS OF BEEF PINEAL TRYPTOPHAN HYDROXYLASE*

Compounds	Concn (M)	Inhibition (%)
2-Propyl-3,4-dihydroxyphenyl acetamide (H-2254)	10^{-4}	85
Norepinephrine	10^{-4}	76
Catechol	10^{-4}	65
Dopamine (3,4-dihydroxyphenylethylamine)	10^{-4}	69
L-Dopa (3,4-dihydroxyphenylalanine)	10^{-4}	62
L-α-Methyldopa	10^{-4}	67
DL-*p*-Chlorophenylalanine	10^{-4}	40
p-Chlorophenylpyruvic acid	10^{-4}	45
p-Chlorophenylacetic acid	10^{-4}	21
Phenylalanine	10^{-3}	66
4-Chlorotryptophan	10^{-3}	45
6-Chlorotryptophan	10^{-3}	44
5-Fluorotryptophan	10^{-3}	33
6-Fluorotryptophan	10^{-3}	58
α-Methyltryptophan	10^{-3}	2
Chloracetyltryptophan	10^{-3}	15
Acetyl-L-tryptophan	10^{-3}	6
7-Aza-tryptophan	10^{-3}	10
DL-α-Methylphenylalanine	10^{-3}	23
α-Methyltyrosine	10^{-3}	23

* The pineal enzyme was prepared as described under Methods. The 40–60 per cent $(NH_4)_2SO_4$ fraction of the 30,000 *g* supernatant was used for the assay; the inhibitor was preincubated for 10 min at 37° with 1·4 mg enzyme before substrate and cofactor were added. The catechol compounds were assayed without addition of exogenous Fe^{++} to the incubation mixture.

DISCUSSION

Characterization of the enzyme, tryptophan hydroxylase, has been hampered by the lack of a soluble and purified enzyme preparation. Indeed, several laboratories have found the enzyme activity to reside only in the particulate fraction[5,7] although more recent reports have described a soluble enzyme with a requirement for a pteridine cofactor.[14] The present work describes a method which yields a clear and partially purified enzyme preparation from the supernatant fraction of rat brainstem and beef pineal homogenates. Studies to further characterize tryptophan hydroxylase were carried out using these preparations.

Purification of the enzyme from the two sources differed with respect to ammonium sulfate fractionation of the supernatant fraction. The pineal enzyme was found to precipitate primarily within a narrow band of 50–60 per cent ammonium sulfate saturation (Table 1), whereas the brainstem enzyme precipitated over a broader range of ammonium sulfate concentration with the 30–60 per cent fraction containing the majority of activity (Table 2). In the case of the pineal enzyme, this yielded a clear enzyme preparation with a high specific activity representing 5-fold purification over the dialyzed homogenate. The brainstem enzyme, although having a lower specific activity, could be obtained in a completely soluble form representing a 2-fold purification with respect to the dialyzed supernatant fraction. While further purification is desirable, this provided an adequate enzyme preparation for a study of enzyme properties. Attempts to further purify the brainstem enzyme utilizing ion exchange resins and other adsorption gels were uniformly unsuccessful. A persistent problem was the fact that after a certain degree of purification was achieved, the enzymatic activity appeared to be inseparable from the majority of the protein regardless of the

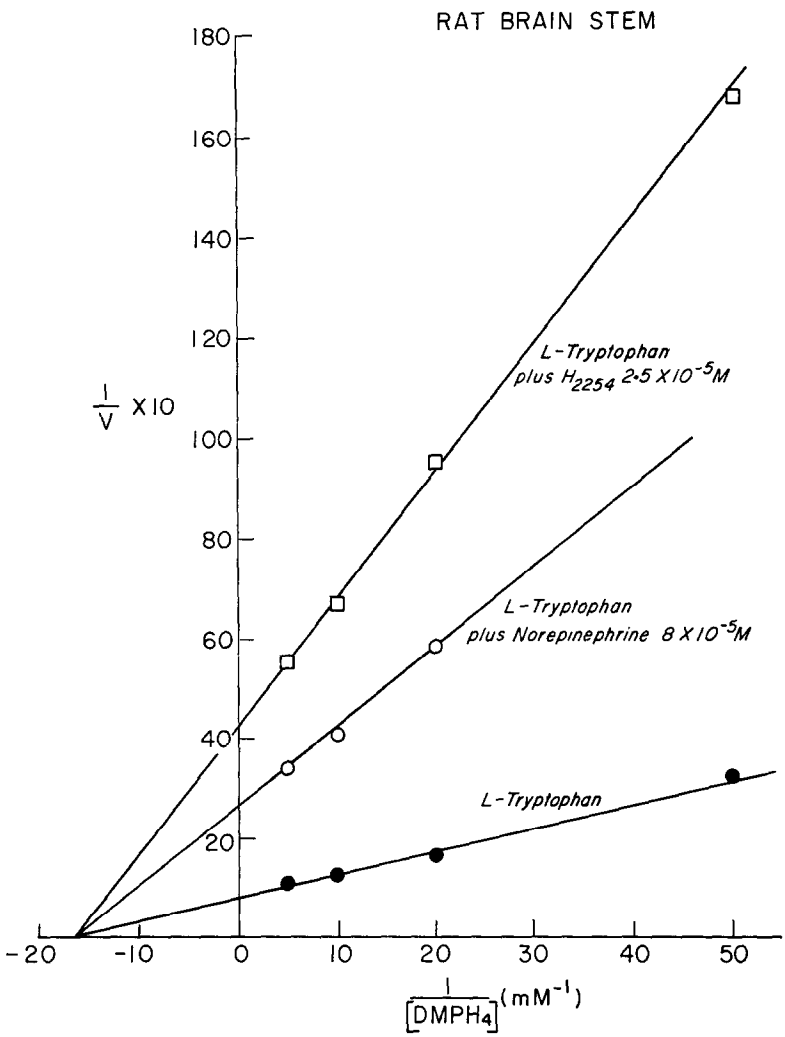

FIG. 5. The inhibition of tryptophan hydroxylase by norepinephrine and H-2254. The reaction mixtures were carried out as described in the text under Methods. Velocity is expressed as mμmoles of 5-HTP formed per mg of protein per hr.

technique employed. This raises the question of whether the enzyme activity is dependent on two proteins, one of which may be nonspecific and inseparable from the body of protein in the enzyme preparation. No specific evidence for such a phenomenon has been obtained.

We have also observed an unexplained variation in activity of different brainstem enzyme preparations despite care in following the purification technique. The values for brainstem tryptophan hydroxylase given in Table 2 represent a level of activity typical for most preparations although occasionally higher activities have been

obtained. The purified enzyme, when stored at 4°, has been found to be reasonably stable for periods up to 1 week. Therefore, it appears doubtful that instability of the brainstem enzyme alone can account for the varying activities we have noted.

Bovine serum albumin was found to stimulate the activity of the crude supernatant and the initial ammonium sulfate fraction from the brainstem. However, it failed to affect the activity of the enzyme preparations after the calcium phosphate gel elution step. It is possible that bovine serum albumin either removes certain inhibitors in the cruder preparations or stabilizes the enzyme. A similar stimulation by bovine serum albumin also has been reported in the case of proline hydroxylase.[16]

The role of iron in aromatic ring hydroxylases is not clear. Tyrosine hydroxylase of adrenal medulla[15] and tryptophan hydroxylase of mast cells[8] are both markedly stimulated by ferrous ions. Liver phenylalanine hydroxylase, while not requiring iron, is strongly inhibited by iron chelators. The iron requirement of tryptophan hydroxylase of brainstem and pineal appears to be more analogous to liver phenylalanine hydroxylase in which the iron requirement is demonstrated mainly by use of iron chelators. The addition of both chelator and ferrous iron in equimolar amounts resulted in a striking enhancement of activity, approx. a 3-fold increase over the control value. Since this enhancement was consistently more than that for added ferrous ion alone, the evidence would suggest that the reaction may be inhibited by other metals which are removed by the chelating agents. The K_m for iron with the pineal enzyme was similar to that for the mast cell tryptophan hydroxylase.[8]

A comparison between the activity of tryptophan hydroxylase and phenylalanine hydroxylase in beef pineal gland and rat brainstem (Table 4) shows an interesting contrast. The pineal enzyme preparation actively hydroxylates phenylalanine, the activity approaching that of tryptophan hydroxylation, although it cannot be concluded that the two activities are associated with a single enzyme. The pteridine cofactor and oxygen are necessary for both reactions. In contrast to pineal, the brainstem preparation has negligible phenylalanine hydroxylase activity. This fact, as well as the difference in purification properties, suggests that the molecular nature of the beef pineal and rat brainstem enzymes may be different. A more extensive study of the enzyme from pineal and brainstem of various species will be required to determine whether this is a species or tissue difference.

The pteridine cofactor specificity for brainstem tryptophan hydroxylase appears to be similar to liver phenylalanine hydroxylase. Both these enzymes show a higher V_{max} and lower K_m with tetrahydrobiopterin than with $DMPH_4$.[8,17] There is good evidence that tetrahydrobiopterin is the naturally occurring factor, at least in the case of liver phenylalanine hydroxylase[17] and the requirement of the purified tryptophan hydroxylase for a pteridine cofactor is consistent with the recent finding of pteridine cofactor-like activity in brain.[18]

The search for inhibitors of tryptophan hydroxylase is important inasmuch as strong inhibitors of this enzyme can be used as tools in the elucidation of the physiological role of serotonin and possibly as therapeutic agents. The study of the inhibition of tryptophan hydroxylase by *p*-chlorophenylalanine both *in vivo* and *in vitro* led to the clinical use of this compound in the carcinoid syndrome.[19,20] Other amino acid analogues, however, have thus far been relatively poor inhibitors. All naturally-occurring and synthetic catechol compounds tested were found to be potent inhibitors of tryptophan hydroxylase. This inhibition, however, was reversible

by the addition of ferrous ion, suggesting that the mechanism of inhibition is through chelation of the metal.

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