ON THE ROLE OF PTERIDINES AS COFACTORS FOR TYROSINE HYDROXYLASE

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Preparations from adrenal medulla and brain contain a specific L-tyrosine hydroxylase, characteristically different from tyrosinase (Nagatsu et al., 1964a). Partially purified preparations of tyrosine hydroxylase from beef adrenal medulla require a tetrahydropteridine for activity (Nagatsu et al., 1964a; Brenneman and Kaufman, 1964). Tetrahydropteridines are similarly required for the hydroxylation of phenylalanine (Kaufman, 1959) and for the oxidation of glyceryl ethers (Tietz et al., 1964). Tetrahydrofolate, 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine, 2-amino-4-hydroxy-6-methyltetrahydropteridine, and reduced biopterin are pteridines which have been shown to stimulate tyrosine hydroxylase activity (Nagatsu et al., 1964a; Brenneman and Kaufman, 1964).

The present communication presents evidence for an apparent structural requirement for pteridines active as cofactors for tyrosine hydroxylase, and observations of the inhibition of tyrosine hydroxylase activity by high concentrations of certain pteridines. In addition, an improved procedure is described which facilitates the assay of large numbers of samples for tyrosine hydroxylase activity by the procedure of Nagatsu et al. (1964b).

MATERIALS AND METHODS

Tyrosine hydroxylase was prepared from homogenates of beef adrenal medulla by the procedure of Nagatsu et al. (1964a). The enzyme was precipitated from the 105,000 x g supernatant by addition

of ammonium sulfate to 40% saturation. The frozen precipitate was used for the experiments reported in this paper.

L-tyrosine-3,5-^{3}H (5360 mc/mmole) was obtained from the New England Nuclear Co. Aliquots were dried under a stream of nitrogen at room temperature and dissolved in water immediately prior to adding to the reaction mixture in order to remove any residual tritiated water.

Tyrosine hydroxylase activity was assayed according to the procedure developed by Nagatsu et al. (1964b) and modified as described below. The standard reaction mixture consisted of 0.1 μmole of L-tyrosine containing 3 to 4 x 10^{4} c.p.m. of L-tyrosine-3,5-^{3}H, 200 μmoles of acetate buffer (pH 6.0), pteridine cofactor in 0.1 ml of 1 M phosphate (pH 7.4) containing 1 M mercaptoethanol, and enzyme in 0.25 ml water. The L-amino acid decarboxylase inhibitor, m-hydroxy-p-bromobenzyloxyamine (NSD-1055, Smith Nephews, Ltd.) was added in 0.1 μmole amounts. The final volume was made to 1.0 ml with water. Upon addition of the enzyme the mixtures were incubated with shaking, in air, at 37°C for 30 minutes, then acidified with 0.05 ml glacial acetic acid and protein separated by centrifugation. The supernatants were transferred by Pasteur pipettes to small columns of Dowex 50 (H^{+}) (0.5 x 3 cm). The precipitates were washed with 1.0 ml water and this was added to the columns after the initial effluent had passed through. The combined effluents (2.0 ml) were collected in a counting vial to which 10 ml of a phosphor solution for scintillation counting (Bray, 1960) had been added. A reagent blank, with no enzyme, was used to correct for the non-enzymatic release of tritium.

To facilitate the use of this procedure for the assay of large numbers of samples, a Lucite rack was prepared which could hold 24 small columns. The columns consisted of disposable Pasteur capillary pipettes (0.5 x 15.0 cm) with straight tips (5 cm) obtained

from Bellco Glass, Inc. These could readily be filled with the required amount of Dowex and stored until used. The supernatants and washes from up to 24 assay samples can then be conveniently transferred to these columns. This transfer procedure has been accomplished for 24 samples in less than one hour in our laboratory.

The reduction of 2-amino-4-hydroxy-6-/trihydroxypropyl-L-erythro/pteridine (neopterin), 2-amino-4-hydroxy-6-methylpteridine, 2-amino-4-hydroxy-7-methylpteridine, and 2-amino-4-hydroxy-6,7-dimethylpteridine to the corresponding tetrahydropteridines was achieved by catalytic hydrogenation in aqueous hydrochloric acid by the method of Pohland et al. (1951). After removal of the platinum dioxide catalyst, the solutions were concentrated to dryness under vacuum at 40°C. The residue from reduced neopterin was stored at 0°C until used, and the other tetrahydropteridines were crystallized from acetone-ethanol-water (3:1:1). The reduced state of all tetrahydropteridines studied was confirmed by ultraviolet absorption spectra in 0.1 N hydrochloric acid.

Dihydrofolate was prepared by the procedure of Futterman (1957). The spectral properties of the dihydrofolate were similar to those described by O'Dell et al. (1947).

Dihydrofolate reductase activity was assayed by measuring the decrease in optical density at 340 mμ of the reaction mixture of 150 μmoles of potassium phosphate buffer (pH 7.5), 0.6 μmole of dihydrofolate, 0.6 μmole of NADPH, and enzyme, at a total volume of 3.6 ml.

RESULTS AND DISCUSSION

Tetrahydrofolate showed slight stimulation of tyrosine hydroxylase activity which increased in the presence of Fe^{++} (Table IA), confirming the results of Nagatsu et al. (1964a). Dihydrofolate did

TABLE I

Effect of Various Pteridines on Tyrosine Hydroxylase Activity

	Pteridine Added	DOPA Formed (mμmoles)
	None	0
A	Tetrahydrofolate	3.6
	Tetrahydrofolate + Fe^{++} (0.5 μmole)	9.0
	Dihydrofolate	0
	N^5-formyltetrahydropteroylglutamate	0
B	Reduced neopterin (2-amino-4-hydroxy-6-trihydroxypropyl-L-erythro-tetrahydropteridine)	24.2
	2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine·HCl	57.1
	2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine thiocyanate	59.1
	2-amino-4-hydroxy-6-methyltetrahydropteridine	71.6
	2-amino-4-hydroxy-7-methyltetrahydropteridine	49.8
C	2-amino-4-hydroxy-5-formyl-6-methyltetrahydropteridine	0
	2-amino-4-hydroxy-5-acetyl-6-methyltetrahydropteridine	0
D	4-methyltetrahydropteridine	0
	5-formyl-4-methyltetrahydropteridine	0
	2-chloro-5-formyl-4-methyltetrahydropteridine	0
	5-formyl-4-dimethylaminotetrahydropteridine	0
	4-dimethylamino-2-methylthio-tetrahydropteridine	0
	5-acetyl-4-dimethylamino-2-methylthio-tetrahydropteridine	0

The assay system was as described in Methods. All pteridines were used at 1 μmole/ml. The amount of enzyme used was that which gave approximately 55% conversion with 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine as cofactor. All reduced compounds are 5,6,7,8-tetrahydropteridines.

not stimulate the enzyme activity (Table IA). This is consistent with the fact that no dihydrofolate reductase activity could be detected in the tyrosine hydroxylase preparation.

The introduction of a formyl group on the 5-position of folate (N^5-formyltetrahydropteroylglutamate) eliminated cofactor activity (Table IA).

Tetrahydroneopterin, a close structural analog of tetrahydrobiopterin, was found to be considerably more active (Table IB) than the conjugated pteridines but less active than the other unconjugated pteridines. Kaufman (1963) has obtained evidence that tetrahydrobiopterin is the natural cofactor for phenylalanine hydroxylase, and has shown it to be more active than the 2-amino-4-hydroxy-6-methyltetrahydropteridine and the 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine in the coupled tyrosine hydroxylase system (Brenneman and Kaufman, 1964). Tietz et al. (1964) found tetrahydroneopterin to be more active than the 2-amino-4-hydroxy-6-methyltetrahydropteridine in the glyceryl ether oxidation system.

Among the other unconjugated tetrahydropteridines (Table IB) the 6-methyl compound was most active. The preparation used was found by NMR spectra to be contaminated with 20% of the less active 7-methyl compound, and thus may be considerably more active when pure. Both the hydrochloride and thiocyanate salts of 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine were very active as cofactors.

The addition of a formyl or an acetyl group at the 5-position of the 2-amino-4-hydroxy-6-methyltetrahydropteridine completely eliminated its cofactor activity (Table IC). Tetrahydropteridines lacking 2-amino and 4-hydroxy groups as well as substituents at the 6- or 7-positions were inactive as cofactors (Table ID).

Our data strongly suggest that the presence of 2-amino and 4-hydroxy groups is a structural requirement for cofactor activity of tetrahydropteridines. The most active tetrahydropteridines contain methyl groups at the 6- and/or 7-positions. The addition of formyl or acetyl groups to the nitrogen in the 5-position of the tetrahydropteridine eliminates cofactor activity, possibly by interferring with the oxidation of the tetrahydropteridine to dihydropteridine.

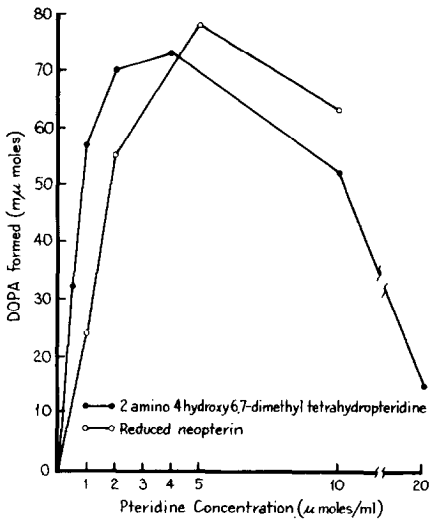

Figure 1 The effect of unconjugated pteridines on tyrosine hydroxylase activity. The assay system was as described in Methods. The volume of enzyme solution used was that which gave approximately 55% conversion of tyrosine-^{3}H to DOPA using 1 μmole of 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine as cofactor. Unconjugated pteridines are defined as those not linked to p-aminobenzoic acid.

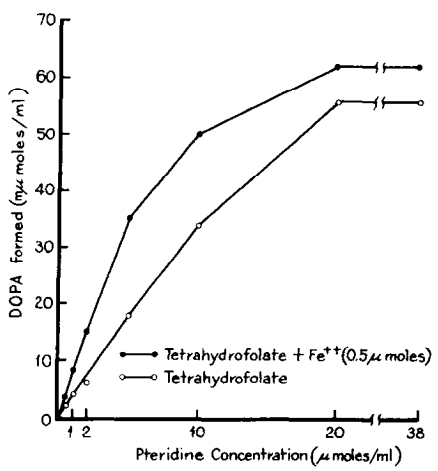

Figure 2 The effect of tetrahydrofolate on tyrosine hydroxylase activity. The assay system was as described in Methods. The volume of enzyme solution used was that which gave approximately 55% conversion of tyrosine-^{3}H to DOPA using 1 μmole of 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine as cofactor.

A surprising observation was the strong inhibition of tyrosine hydroxylase activity by 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine and reduced neopterin at concentrations of 10 and 20 μmoles/ml (Figure 1). The 2-amino-4-hydroxy-6-methyltetrahydropteridine derivative also demonstrated this inhibition. Tetrahydrofolate showed no inhibition when present in concentrations as high as 38 μmoles/ml, although maximum activity was reached at 20 μmoles/ml (Figure 2). The addition of Fe^{++} to tetrahydrofolate enhanced its cofactor activity, but no inhibition was observed at high tetrahydrofolate concentrations (Figure 2).

The inhibition observed by high concentrations (20 μmoles/ml) of 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine was completely reversed by the addition of Fe^{++} (Table II). At low pteridine

TABLE II

Effect of Fe^{++} and o-Phenanthroline on Tyrosine Hydroxylase Activity at High and Low Concentrations of 2-Amino-4-hydroxy-6,7-dimethyltetrahydropteridine ($DMPH_4$)

Fe^{++} (1 μm/ml)	o-Phenanthroline (5 μm/ml)	DOPA Formed (mμmoles) $DMPH_4$ (1 μm/ml)	DOPA Formed (mμmoles) $DMPH_4$ (20 μm/ml)
-	-	35.4	8.8
+	-	60.4	57.3
-	+	0	0

The assay system was as described in Methods. All samples contained 10% methanol which lowered enzymatic conversion to 35% with 1 μm/ml $DMPH_4$. o-Phenanthroline was dissolved in methanol.

concentration (1 μmole/ml), Fe^{++} markedly stimulated activity. In the absence of added Fe^{++}, o-phenanthroline completely inhibited activity at both high and low concentrations of pteridine (Table II). These data strongly suggest an Fe^{++} requirement for tyrosine hydroxylase.

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