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EFFECTS OF STRUCTURES OF TETRAHYDROPTERIN COFACTORS ON RAT BRAIN TRYPTOPHAN HYDROXYLASE

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Summary

Effects of structures of the side chain at position 6 of 12 tetrahydropterin cofactors including 4 stereoisomers of tetrahydrobiopterin and tetrahydroneopterin on the activity of rat brain tryptophan hydroxylase were examined. Tetrahydrobiopterins and tetrahydroneopterins having a side chain of a L-*erythro* or D-*threo* configuration showed lower K_m values for both the pterin cofactor and tryptophan substrate and also higher V values than their enantiomers. L-*erythro*-Tetrahydrobiopterin had the highest cofactor activity among all the pterin cofactors examined. Since reduction of biopterin to tetrahydrobiopterin introduces another center of asymmetry at 6-position of the pterin ring, L-*erythro*-tetrahydrobiopterin obtained by chemical reduction is a mixture of two diastereoisomers. The two diastereoisomers of L-*erythro*-tetrahydrobiopterin, i.e. (6*R*)-L-*erythro*-tetrahydropterin and the (6*S*)-isomer were separated by high-performance liquid chromatography, and were examined for their cofactor activity. The two diastereoisomers gave similar K_m values toward pterin itself and toward tryptophan, but the natural (6*R*)-isomer gave much higher V values than the (6*S*)-isomer; the (6*S*)-isomer was nearly inactive for rat brain tryptophan hydroxylase because of its very low V value. These results support the hypothesis that (6*R*)-L-*erythro*-tetrahydrobiopterin may be the natural cofactor of rat brain tryptophan hydroxylase.

Introduction

Tryptophan hydroxylase (L-tryptophan, tetrahydropteridine:oxygen oxidoreductase (5-hydroxylating), EC 1.14.16.4) [1—4], which catalyzes the

hydroxylation of L-tryptophan to 5-hydroxy-L-tryptophan, is generally accepted as the rate-limiting enzyme in the biosynthesis of the neurotransmitter, serotonin (5-hydroxytryptamine). Since L-*erythro*-tetrahydrobiopterin is the natural cofactor of phenylalanine hydroxylase [5], the compound is supposed to be also the natural cofactor of tryptophan hydroxylase. We recently synthesized four isomeric biopterins, i.e. 6-L-*erythro*, 6-D-*erythro*-, 6-L-*threo*-, and 6-D-*threo*-(1,2-dihydroxypropyl)pterins [6], and studied the cofactor activities on their 5,6,7,8-tetrahydro derivatives for tyrosine hydroxylase activity [7]. It was found that the kinetic properties of the isomeric tetrahydropterins were dependent much on the configuration at C-1′ (the carbon nearest to the pteridine ring) of the side chain. Both L-*erythro*- and D-*threo*-tetrahydrobiopterins, which have the same L-configuration at C-1′ exhibited similar cofactor characteristics, and showed possible regulatory mechanisms such as substrate inhibition and two different K_m values toward the pterin cofactor. On the other hand, the D-*erythro*- and L-*threo*-isomers, which have the same D-configuration at C-1′ behaved in a similar fashion as cofactor and lacked such regulatory properties [7].

In the chemical reduction of 6-substituted pterin to 5,6,7,8-tetrahydro derivatives, a new asymmetric center is introduced at the 6-position resulting in the formation of two diastereomers. It has been reported that the enzymatically reduced L-*erythro*-tetrahydrobiopterin had *R*-configuration at the 6-position and showed a higher cofactor activity for both bovine adrenal tyrosine hydroxylase and bovine pineal tryptophan hydroxylase than its (6*S*)-diastereomer [8]. The above mentioned regulatory mechanisms on tyrosine hydroxylase were demonstrated only with the natural (6*R*)-isomer [8]. An analogous regulatory mechanism, i.e. substrate (L-phenylalanine) inhibition and two K_m values, on bovine pineal tryptophan hydroxylase [9] was observed only with (6*R*)-L-*erythro*-tetrahydrobiopterin [8]; (6*S*)-isomer was less active and did not show any regulation mechanism. Significant differences have also been observed between the two diastereoisomers on regulatory properties of phenylalanine hydroxylase [10].

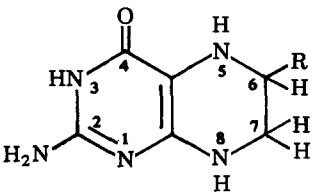
Friedman et al. [4] reported that rabbit hind brain tryptophan hydroxylase showed a lower K_m value for tryptophan in the presence of tetrahydrobiopterin than in the presence of a pseudocofactor, 6,7-dimethyltetrahydropterin. However, the effects of the stereochemical structure of the side chain of 6-substituted tetrahydropterins on the enzyme have not been studied yet.

This study has been undertaken to compare the kinetic properties of tryptophan hydroxylase from rat brain stem with various stereochemically isomeric tetrahydropterins as cofactors to previously studied tyrosine hydroxylase. Furthermore, the cofactor activities of (6*R*)- and (6*S*)-diastereoisomers of L-*erythro*-tetrahydrobiopterin for tryptophan hydroxylase have been examined to clarify the effect of the configurations at C-6 and C-1′ position on the cofactor properties.

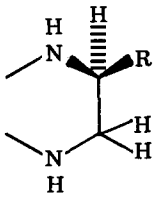
Materials and Methods

Partisil-SCX column for high-performance liquid chromatography was obtained from Whatman. The following pterins required for the synthesis of

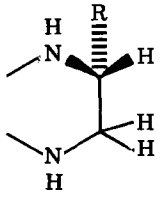
the twelve tetrahydropterins examined in this study were synthesized in our laboratory according to the known methods: the four isomers of biopterin [6], 6-[L-(and D)-1′,2′-dihydroxyethyl]pterins [11], four isomers of neopterin [12], 6-methylpterin [13], and 6-hydroxymethylpterin [14]. The purity of the pterins was confirmed by their ultraviolet spectra, by paper chromatography, and by permanganate oxidation to the known pterin-6-carboxylic acid [15]. The corresponding 5,6,7,8-tetrahydro derivative of each pterin was prepared by catalytic hydrogenation in 0.1 M HCl using platinum oxide as catalyst [16]. The reduction was followed by examining the ultraviolet absorption spectrum. After completion of the reduction, the catalyst was removed by filtration, and the filtrate containing tetrahydropterin was sealed without air and stored at −20°C in the dark. The structures of these 12 tetrahydropterins are shown in Scheme I. The molar concentration of these tetrahydropterins were estimated



Tetrahydropterin

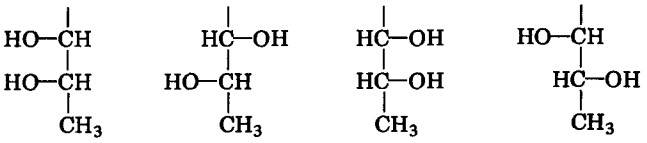


6*R*



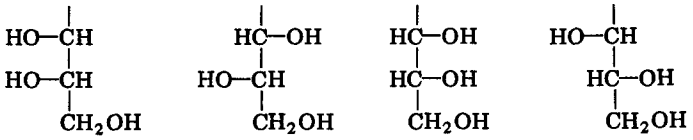
6*S*

Biopterin: R =



L-*erythro* L-*threo* D-*erythro* D-*threo*

Neopterin: R =

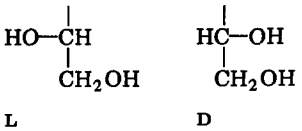


L-*erythro* L-*threo* D-*erythro* D-*threo*

6-Methyl: R =

6-Hydroxymethyl: R =

Dihydroxyethyl: R =



L D

Scheme I. Structures of tetrahydropterins.

based on the extinction coefficient of 18 500 $M^{-1} \cdot cm^{-1}$ at 264 nm in 2 M HCl. The two diastereoisomers of L-*erythro*-tetrahydrobiopterin [(6*R*) and (6*S*)] were obtained from the above prepared tetrahydrobiopterin by high-

performance liquid chromatography on Partisil-SCX according to the method of Bailey and Ayling [10].

Rats were killed by decapitation, and the brain stem (thalamus, hypothalamus, midbrain, pons and medulla) was quickly separated from other tissues. The brain stem was homogenized in 3 volumes (v/w) of 0.05 M Tris-acetate buffer, pH 7.5, containing 2 mM dithiothreitol. The homogenate was then centrifuged at 39 000 × *g* for 60 min and the supernatant was used as the enzyme source.

Tryptophan hydroxylase activity was assayed by the method of Friedman et al. [4] with slight modification. The incubation mixture (total volume 500 μl) contained 120 mM Tris-acetate (pH 7.5), 1 mM dithiothreitol, 1 mM NSD-1055, 75 μg catalase, 5—1000 μM L-tryptophan, 20—1000 μM tetrahydropterin, and 40—100 μl enzyme. For the control, D-tryptophan instead of L-tryptophan was added to the incubation mixture. After incubation at 37°C for 25 min with shaking in air (20.9% oxygen), the reaction was stopped by the addition of 50 μl 60% $HClO_4$, the mixture was centrifuged at 2500 rev./min for 10 min and 400 μl of the supernatant were transferred into a test tube containing 100 μl concentrated HCl. The fluorescence at 527 nm of the solution was determined on a Shimadzu RF-500 spectrophotofluorimeter with an excitation at 295 nm. The amount of 5-hydroxytryptophan formed was calculated from a standard curve of authentic 5- hydroxytryptophan.

The K_m values and maximal velocities were determined from Lineweaver-Burk plots using Wilkinson's program [17].

Results

K_m value of each tetrahydropterin as cofactor

The K_m value of each tetrahydropterin cofactor was determined from the Lineweaver-Burk plots (Fig. 1) obtained by studying the kinetics in air (20.9% oxygen) in the presence of 100 μM L-tryptophan as substrate. As shown in Table I and Fig. 1, L-*erythro*- and D-*threo*-tetrahydrobiopterins showed similar K_m values which were significantly lower than those for D-*erythro*- and L-*threo*-tetrahydrobiopterins. Similar results were also given by the four tetrahydroneopterin isomers as cofactor (Table I). Although we previously reported that in the kinetic studies on tyrosine hydroxylase L-*erythro*- and D-*threo*-tetrahydrobiopterin showed two K_m values depending on the concentrations of the tetrahydropterin [7], the two pterins showed a classical Michaelis-Menten curve for rat brain tryptophan hydroxylase and gave a single K_m value.

6-Methyl-, 6-hydroxymethyl-, 6-L-dihydroxyethyl- and 6-D-dihydroxyethyltetrahydropterin gave much higher K_m values than L-*erythro*-tetrahydrobiopterin.

The K_m value of 6-(L-dihydroxyethyl)tetrahydropterin, which has the same L-configuration at C-1′ as L-*erythro*- or D-*threo*-tetrahydrobiopterin, was also significantly lower than that of 6-(D-dihydroxyethyl)tetrahydropterin which has the reversed C-1′ configuration. Inhibition by high concentrations of the pterin cofactor was observed only with four tetrahydrobiopterins and 6-methyltetrahydropterin.

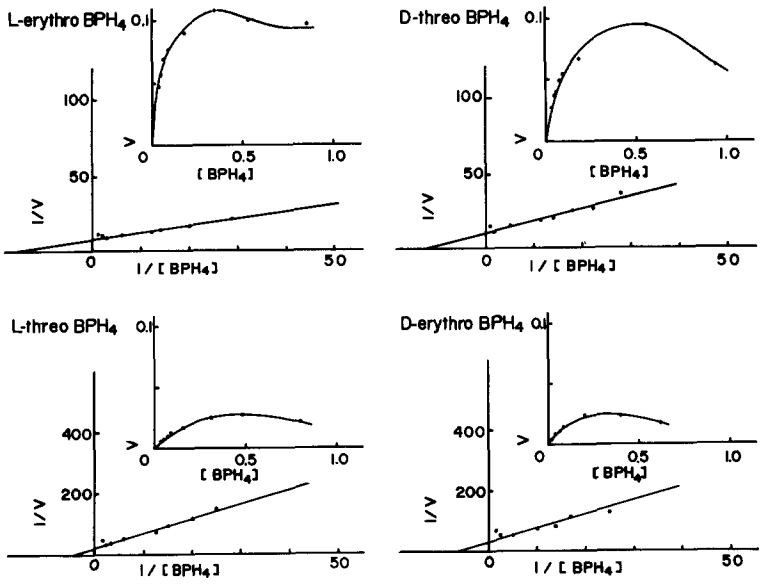


Fig. 1. Lineweaver-Burk and Michaelis-Menten plots illustrating the effects of L-*erythro*-, D-*threo*-, L-*threo*-, and D-*erythro*-tetrahydrobiopterin on the rate of 5-hydroxytryptophan formation by rat brain tryptophan hydroxylase. Incubation was done in air (20.9% oxygen) using 100 μM L-tryptophan. Enzyme activity is expressed as nmol 5-hydroxytryptophan formed per min per mg protein. [BPH_4] is in mM. Reciprocal units for Lineweaver-Burk plots.

K_m value for tryptophan with each tetrahydropterin as cofactor

The K_m value of tryptophan was measured with each tetrahydrobiopterin cofactor at 100 μM and with each of the other tetrahydropterin cofactors at 300 μM in air (20.9% oxygen). Tetrahydrobiopterin isomers except L-*threo*-isomer gave much lower K_m values for tryptophan than the corresponding tetrahydroneopterins and other tetrahydropterins examined. L-*threo*-Tetrahydrobiopterin gave anormalously high K_m values than the other three tetrahydropterin isomers. The same tendency was also observed in neopterin isomers where the L-*threo*-isomer gave higher K_m than the other isomers (Table I). As shown in Fig. 2, tryptophan was inhibitory at concentrations higher than 200 μM only with L-*erythro*- or D-*threo*-tetrahydrobiopterin as cofactor. The K_m value for tryptophan with 6-(L-dihydroxyethyl)tetrahydropterin as cofactor was lower than that with the D-isomer as cofactor.

Maximal velocity (V) of tryptophan hydroxylase with each tetrahydropterin as cofactor

The apparent V values were determined from the Lineweaver-Burk plots by extraporating the substrate concentration to an infinite in the presence of each tetrahydropterin cofactor at 100 μM and air (oxygen 20.9%), and also by extraporating the cofactor concentration to an infinite in the presence of 100 μM tryptophan and air (Fig. 1, Table I).

Among the four isomeric tetrahydrobiopterins, the L-*erythro* isomer gave the highest V value, followed by the D-*threo*-, L-*threo*-, and D-*erythro*-tetrahydrobiopterins in a decreasing order of activity. 6-Methyl-tetrahydropterin have a

TABLE I

V AND K_m VALUES OF TETRAHYDROPTERINS AND TRYPTOPHAN WITH EACH TETRAHYDROPTERIN AS COFACTOR OF TRYPTOPHAN HYDROXYLASE

Tryptophan hydroxylase activity was assayed as described in Materials and Methods with each tetrahydropterin as cofactor. K_m values of each tetrahydropterin and tryptophan were determined from Lineweaver-Burk plots using 100 μM tryptophan or 100 μM each tetrahydrobiopterin and 300 μM each of the other tetrahydropterins in air using the Wilkinson's program [17] and expressed as mean ± S.E. V values were determined from Lineweaver-Burk plots using various concentrations of tryptophan with 100 μM each tetrahydrobiopterin and with 300 μM each of the other tetrahydropterins, or using various concentrations of each tetrahydropterin with 100 μM tryptophan. 5-Hydroxytryptophan formed from tryptophan was assayed fluorimetrically [4].

Tetrahydropterin	K_m for tetrahydropterin (μM)	K_m for tryptophan (μM)	V value (pmol/min per mg protein)	
			Tetrahydropterin →∞	Tryptophan →∞
L-*erythreo*-Tetrahydrobiopterin	57 ± 5	48 ± 7	126 ± 4	139 ± 9
D-*threo*-Tetrahydrobiopterin	81 ± 12	64 ± 8	100 ± 7	75 ± 5
L-*threo*-Tetrahydrobiopterin	190 ± 31	151 ± 33	39 ± 4	34 ± 4
D-*erythro*-Tetrahydrobiopterin	140 ± 26	63 ± 4	32 ± 4	13 ± 1
L-*erythreo*-Tetrahydroneopterin	87 ± 16	260 ± 36	17 ± 1	45 ± 3
D-*threo*-Tetrahydroneopterin	74 ± 10	246 ± 30	18 ± 1	42 ± 2
L-*threo*-Tetrahydroneopterin	126 ± 5	455 ± 44	12 ± 1	36 ± 2
D-*erythreo*-Tetrahydroneopterin	111 ± 15	294 ± 51	13 ± 1	27 ± 3
6-(L-Dihydroxyethyl) tetrahydropterin	184 ± 14	145 ± 29	57 ± 22	61 ± 7
6-(D-Dihydroxyethyl) tetrahydropterin	523 ± 103	276 ± 34	42 ± 6	42 ± 3
6-Hydroxymethyltetrahydropterin	340 ± 85	188 ± 4	56 ± 7	70 ± 7
6-Methyltetrahydropterin	191 ± 18	152 ± 13	112 ± 6	129 ± 5

high V value comparable to that with L-*erythro*- or D-*threo*-tetrahydrobiopterin. With other tetrahydropterins as cofactor, the V values were low.

Reactivities of (6R)- and (6S)-L-erythro-tetrahydrobiopterin with rat brain tryptophan hydroxylase

The chemical reduction of 6-substituted pterins to the tetrahydro derivatives introduces another center of asymmetry at position 6, and all of the above studies were carried out using a mixture of (6*R*)- and (6*S*)-isomers of each tetrahydropterin as cofactor. However, such isomers were expected to be of different cofactor characteristics, since the tridimensional orientation of the 6-side chain could affect the binding of the cofactor to the enzyme. We, therefore, separated the (6*R*)- and (6*S*)-diastereoisomers of L-*erythro*-tetrahydrobiopterin by high-performance liquid chromatography on Partisil-SCX, and examined their kinetic properties. The results are shown in Table II. The experiments were carried out in air by changing the concentrations of (6*R*)- or (6*S*)-L-*erythro*-tetrahydrobiopterin cofactor in the presence of 100 μM L-tryptophan as substrate, or by changing the concentrations of L-tryptophan in the presence of 100 μM (6*R*)- or (6*S*)-L-*erythro*-tetrahydropterin cofactor.

Unexpectedly, (6*R*)- and (6*S*)-L-*erythro*-tetrahydrobiopterin showed comparable K_m values for both of the cofactor and substrate with rat brain

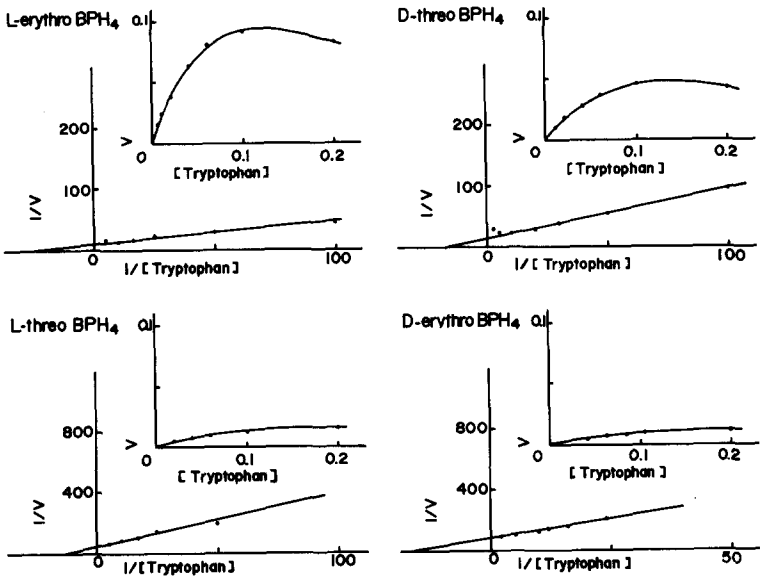


Fig. 2. Lineweaver-Burk and Michaelis-Menten plots illustrating the effect of tryptophan with L-*erythro*-, D-*threo*-, L-*threo*-, and D-*erythro*-tetrahydrobiopterin as cofactor on the rate of 5-hydroxytryptophan formation by rat brain tryptophan hydroxylase. Incubation was done in air (20.9% oxygen) using 100 μM tetrahydrobiopterin as cofactor. Enzyme activity (v) is expressed as nmol 5-hydroxytryptophan formed per min per mg protein. [Tryptophan] is in mM.

tryptophan hydroxylase. However, a remarkable difference between the (6*R*)- and (6*S*)-diastereomers was found in the V values, where the maximal velocity with (6*R*)-L-*erythro*-tetrahydrobiopterin as cofactor was much higher than that with (6*S*)-diastereoisomer as cofactor. In contrast to tyrosine hydroxylase or bovine pineal tryptophan hydroxylase which showed two K_m values for (6*R*)-L-*erythro*-tetrahydrobiopterin, the present studied enzyme, rat brain tryptophan hydroxylase, showed a single K_m value for the cofactor.

TABLE II

V AND K_m VALUES OF (6*R*)- AND (6*S*)-L-*erythreo*-TETRAHYDROBIOPTERINS AND TRYPTOPHAN WITH EACH TETRAHYDROBIOPTERINS AS COFACTOR OF TRYPTOPHAN HYDROXYLASE

Tryptophan hydroxylase activity was assayed as described in Materials and Methods with each L-*erythro*-tetrahydrobiopterin as cofactor. K_m values of each tetrahydropterin and of tryptophan were determined from Lineweaver-Burk plots using 100 μM tryptophan or 100 μM tetrahydrobiopterin in air using Wilkinson's program [17] and expressed as mean ± S.E. V values were determined from Lineweaver-Burk plots using various concentrations of each tetrahydrobiopterin with 100 μM L-tryptophan. 5-Hydroxytryptophan formed from L-tryptophan was determined fluorimetrically [4].

Tetrahydrobiopterin	K_m for BPH_4 (μM)	K_m for Trp (μM)	V value (pmol/min per mg protein)	
			$BPH_4 \rightarrow \infty$	Trp $\rightarrow \infty$
(6*R*)-L-*erythro*-tetrahydrobiopterin	47 ± 5	48 ± 10	135 ± 5	136 ± 12
(6*S*)-L-*erythro*-tetrahydrobiopterin	39 ± 8	59 ± 11	13 ± 1	15 ± 1

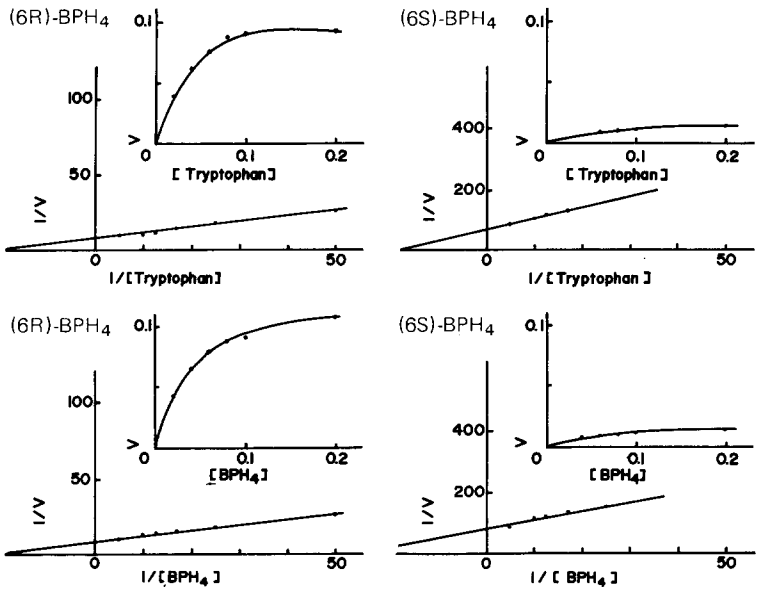


Fig. 3. Lineweaver-Burk and Michaelis-Menten plots illustrating the effect of (6*R*)- or (6*S*)-L-*erythro*-tetrahydrobiopterin on the rate of 5-hydroxytryptophan formation by rat brain tryptophan hydroxylase. Incubation was done in air (20.9% oxygen) using 100 μm L-tryptophan. Enzyme activity is expressed in nmol 5-hydroxytryptophan formed per min per mg protein. [BPH_4] is in mM.

Discussion

Among the four isomeric tetrahydrobiopterins, L-*erythro*- and D-*threo*-isomers revealed similar kinetic properties. The D-*erythro*- and L-*threo* isomers were also similar as cofactor, but these compounds were less active than the former two cofactor (Fig. 1); the V value with L-*erythro*- or D-*threo*-isomer as cofactor was higher than that with D-*erythro*- or L-*threo*-isomer, and the K_m values for L-*erythro*- and D-*threo*-isomers were lower than those for D-*erythro*- or L-*threo*-isomers. The kinetic properties of the pterin cofactor are much affected by the configuration of the hydroxyl groups, especially by the configuration at C-1′ of the side chain; L-*erythro*- and D-*threo*-tetrahydrobiopterins have the same L-configuration at C-1′, and D-*erythro*- and L-*threo*-isomers have the same D-configuration at C-1′. The above results indicate that the L-configuration at C-1′ is more favorable for a high cofactor activity than the opposing D-configuration. The same tendency was also observed with tetrahydroneopterins and even more evident with 6-(L- and 6-D-dihydroxyethyl)tetrahydropterins (Table I). (L-Dihydroxyethyl)tetrahydropterin gave lower K_m values toward pterin cofactor itself and tryptophan and higher V values than (D-dihydroxyethyl)tetrahydropterin. A significant substrate inhibition was observed when L-*erythro*- or D-*threo*-tetrahydrobiopterin was used as cofactor. These results are consistent with the previous result observed on bovine adrenal tyrosine hydroxylase, which further support the present concept that tryptophan hydroxylase is more active with the cofactors having L-configuration at C-1′ of the side chain than with the cofactors having D-configuration. Since

L-*erythro*-tetrahydrobiopterin is the most active cofactor for tryptophan hydroxylase among all the pterin cofactors examined, this compound could also be the natural cofactor of tryptophan hydroxylase. However, there are significant differences between bovine adrenal tyrosine hydroxylase and rat brain tryptophan hydroxylase in their kinetic properties toward the four stereoisomeric tetrahydrobiopterins. We had reported that L-*erythro*- or D-*threo*-tetrahydrobiopterin gave slightly higher maximum velocity for tyrosine hydroxylase than D-*erythro*- and L-*threo*-tetrahydrobiopterins and had two distinct K_m values toward the pterin cofactor. In contrast, with rat brain tryptophan hydroxylase, L-*erythro*- and D-*threo*-tetrahydrobiopterin gave single K_m values and much higher V values compared to other tetrahydropterins as cofactor. On tyrosine hydroxylase, no inhibition was observed with tetrahydropterins, but in the present study, a weak inhibition with tetrahydrobiopterin isomers was observed on brain tryptophan hydroxylase. Similar inhibition with tetrahydropterins has been reported with rabbit brain tryptophan hydroxylase [4].

By the chemical reduction of a 6-substituted pterin, an asymmetrical center is introduced at position 6 resulting in the formation of two diasteromers. Recently Bailey and Ayling [10] separated the 6-diasteromers of L-*erythro*-tetrahydrobiopterin by high-performance liquid chromatography and compared the two diasteromers as cofactor of phenylalanine hydroxylase. They found that the K_m value toward natural (6*R*)-L-*erythro*-tetrahydrobiopterin was comparable to the K_m value toward the unnatural (6*S*)-isomer, but the V value with the natural isomer was four times higher than that with the unnatural isomer. In the present study, we also found that (6*R*)-L-*erythro*-tetrahydrobiopterin and the (6*S*)-isomer gave comparable K_m values toward both the substrate and the cofactor but V value with (6*R*)-L-*erythro*-tetrahydrobiopterin was about 10 times higher than that with (6*S*)-L-*erythro*-tetrahydrobiopterin. However, (6*R*)- and (6*S*)-L-*erythro*-tetrahydrobiopterins with bovine pineal tryptophan hydroxylase showed almost the same V values but very different K_m values: the (6*R*)-isomer showed 10 or 20 times lower K_m values than the (6*S*)-isomer [8]. In this respect, kinetic properties of rat brain tryptophan hydroxylase are different from those of bovine tryptophan hydroxylase and similar to those of phenylalanine hydroxylase.

From these results, we conclude that for cofactor activity with rat brain tryptophan hydroxylase the configuration at the 6-position of tetrahydrobiopterin as well as the configuration at C-1′ of the side chain are important as with bovine adrenal tyrosine hydroxylase. The cofactor activity is highest when C-6 has *R*-configuration and C-1′ has L-configuration. The kinetic properties of rat brain tryptophan hydroxylase are different in several aspects to those of bovine adrenal tyrosine hydroxylase [7] or bovine pineal tryptophan hydroxylase [8], especially in that the natural (6*R*)-isomer gave much higher V than the (6*S*)-isomer which was nearly inactive for rat brain tryptophan hydroxylase.

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isomers of L-*erythro*-tetrahydrobiopterin by high-performance liquid chromatogarphy.

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