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CHARACTERISTICS OF ENZYMIC DECARBOXYLATION OF L-THREO-3,4-DIHYDROXYPHENYLSERINE USING HOG RENAL L-AROMATIC AMINO ACID DECARBOXYLASE

CHIYOKO INAGAKI and CHIKAKO TANAKA*
Department of Pharmacology, Faculty of Medicine, Kyoto University, Kyoto 606, Japan

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Abstract—Decarboxylation of L-threo-3,4-dihydroxyphenylserine (L-threo-DOPS) was examined *in vitro* using hog renal L-aromatic amino acid decarboxylase. Decarboxylation was measured by chemical assay of the reaction product, noradrenaline (NA), and was found to occur specifically with the L-isomer and not with the D-isomer. The maximum rate of decarboxylation of L-threo-DOPS was obtained in the presence of 10 μM pyridoxal phosphate (PALP) at 37° and pH 8.6. Under the optimal conditions, K_m and V_{max} for L-threo-DOPS were 4×10^{-4} M and 2.80 nmoles NA/mg of protein/min respectively. Decarboxylation of L-threo-DOPS was competitively inhibited by L-3,4-dihydroxyphenylalanine (L-DOPA) and L-5-hydroxytryptophan (L-5-HTP). D-Threo-DOPS and D-DOPA at 0.125 mM inhibited the decarboxylation of L-threo-DOPS to about half that of the control. D-Erythro-DOPS at higher concentrations had a slight inhibitory effect on the decarboxylation of L-threo-DOPS, while D-5-HTP and D-tryptophan up to 1 mM had no effect whatever. The inhibition induced by D-threo-DOPS was competitive when the concentration of D-threo-DOPS was below that of L-threo-DOPS. When the concentration of the D-isomer was higher than that of the L-isomer, a noncompetitive inhibition was observed. Inhibition due to inactivation of PALP by interaction of PALP and D-isomer was ruled out. These results suggest that inhibition of decarboxylation of L-threo-DOPS by D-threo-DOPS is the result of the interaction of the D-isomer with the enzyme.

3,4-Dihydroxyphenylserine (DOPS) has four stereoisomers, i.e. L-threo, D-threo-, L-erythro- and D-erythro-DOPS. Racemic threo-DOPS has been reported to be decarboxylated by mammalian tissues and to form natural noradrenaline (NA) *in vitro* and *in vivo* [1, 2], while decarboxylation of racemic erythro-DOPS has been considered to yield unnatural NA [3, 4]. Recently, it has been demonstrated that the L-isomer of threo-DOPS is readily decarboxylated to l-NA in the brain and heart in the rat, while D-threo-DOPS is not decarboxylated to a major extent [5, 6]. We reported the conversion of L-threo-DOPS to natural NA by incubation of the amino acid with the supernatant fraction (8000 *g*, 10 min) of rat kidney homogenate [7, 8]. Studies on L-threo-DOPS have thus proven it to be an effective precursor of natural NA and a sterically characteristic substrate of L-aromatic amino acid decarboxylase. However, there is insufficient information concerning the enzymic decarboxylation of this amino acid. In the present work, we used hog kidney enzyme in an attempt to elucidate the enzymological features of decarboxylation of L-threo-DOPS.

METHODS

Enzyme preparation. L-Aromatic amino acid decarboxylase was prepared from hog kidneys by ammonium sulfate fractionation. All steps in the purification were carried out at 0–5° with reference to the method of Christenson *et al.* [9]. The cortical layer cut from a defatted kidney was minced and homogenized in a Teflon–glass homogenizer with 4 volumes of 10 mM Tris–HCl (pH 7.4), containing 10 mM 2-mercaptoethanol. The homogenate was centrifuged at 20,000 *g* for 30 min, and the supernatant was filtered through two layers of cheesecloth. A saturated ammonium sulfate solution adjusted to pH 7.4 was added dropwise to the stirred supernatant to bring it to a 32% saturation. The solution was stirred for another 20 min and recentrifuged at 20,000 *g* for 20 min, after which the precipitate was discarded. The supernatant was brought to a 49% saturation point by the dropwise addition of a saturated ammonium sulfate solution, and was then stirred for 20 min and centrifuged as before. The precipitate was resuspended in approximately 0.5 vol. (of original tissue) of 10 mM Tris–HCl (pH 7.4), containing 10 mM 2-mercaptoethanol. The solution was dialyzed against 10 mM Tris–HCl (pH 7.4) for 3 hr, divided into 2-ml aliquots and stored at −20°. Protein concentration was determined by the method of Lowry *et al.* [10].

Decarboxylation of DOPS. The enzyme reaction was carried out in duplicate at 37° in 2 ml medium consisting of 125 mM Tris–HCl (pH 8.6), 20 μM pargyline, 10 μM pyridoxal phosphate (PALP), 0.5 mM L-threo-DOPS and 1.0 mg of enzyme protein except as indicated otherwise. The reaction was started by the addition of 0.1 ml of enzyme solution after a 5-min preincubation of the medium (1.9 ml), and stopped at 10 or 20 min of incubation time by the addition of 4 ml of ice-cold 0.4 N perchloric acid with 10 mg sodium metabisulfite and 200 mg ethy-

* Present address: Department of Pharmacology, Kobe University, School of Medicine, Ikuta-ku, Kobe 650, Japan.

lenediamine tetraacetic acid disodium salt (EDTA). The mixture was first cooled in ice and then centrifuged at 5000 *g* for 10 min. The supernatant was decanted into a glass beaker containing 20 ml of ice-cold 0.4 N perchloric acid, 1 ml of 100 mM EDTA–Tris (pH 7.0) and 10 mg sodium metabisulfite. The mixture was adjusted from pH 4.0 to 4.2 with 2 N NaOH and passed through a Dowex 50 X4 column (0.7 cm in diameter, wet resin volume 1 ml) previously washed and pH adjusted by subsequent pass of 5 ml of 1 N NaOH, 5 ml of distilled water, 20 ml of 2 N HCl, 5 ml of distilled water, 10 ml of 1 N sodium acetate buffer (pH 6.0) and 5 ml of distilled water. After rinsing the column three times with 5 ml, 20 ml and further 20 ml of distilled water, elution was performed with 10 ml of 1 N HCl. The eluate was adjusted from pH 6.0 to 6.2 by 5 N sodium carbonate, and an aliquot of 2.0 ml was taken for assay. One ml of 0.1 M potassium phosphate buffer (pH 6.5), 0.1 ml of zinc sulfate solution (0.25 g $ZnSO_4$ 7 H_2O/100 ml of water) and 5.8 ml of distilled water were added. Oxidation was performed by addition of 0.1 ml of 0.25% potassium ferricyanide for 2 min, after which 1 ml of 0.5% ascorbic acid in 5 N NaOH was added. After 5 min, the fluorescence of the sample was read in an Aminco–Bowman spectrofluorometer. The uncorrected fluorescence peaks of excitation and emission were at 400 and 506 nm respectively. NA (0.5 and 1.0 μg) treated in the same way as the sample served as the internal standard. Recovery of NA was 80–92 per cent. DOPS even at the highest concentration did not interfere with the assay.

Decarboxylation of 5-hydroxytryptophan. The reaction was carried out at 37° in 2 ml medium containing 125 mM Tris–HCl (pH 9.0), 0.2 mM pargyline, 0.1 mM pyridoxal phosphate, 1 μM–1 mM L-5-hydroxytryptophan (L-5-HTP) and 1 mg of enzyme protein. The reaction was started by the addition of enzyme and stopped by placing the test tube containing the reaction mixture in a boiling water bath for 1 min. The tube was cooled in ice and the content diluted to 5 ml with distilled water. 5-Hydroxytryptamine (5-HT) in this solution was separated from 5-HTP according to the procedures described by Lovenberg *et al.* [11]. The native fluorescence of 5-HTP was measured in an Aminco–Bowman spectrofluorometer, the uncorrected excitation and emission wavelengths being 290 and 335 nm, respectively. Decarboxylation of 5-HTP was represented as nmoles 5-HT/mg of protein/min. All experiments were done in duplicate. Each figure is the representative of 2–4 experiments.

Materials. L-Threo-, D-threo- and D-erythro-DOPS were synthesized and purified in the Laboratory of Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan. Each purified threo-isomer had a negative rotation of $[\alpha]_D^{20} = -42.6$ ($c = 1$, 1 N HCl) (purity: 99.5 per cent) or a positive rotation of $[\alpha]_D^{20} = +43.2$ ($c = 1$, 1 N HCl) (purity: 99.8 per cent) respectively. Contamination by NA assayed in our laboratory was found to be 0.009 per cent in L-threo-DOPS, 0.011 per cent in D-threo-DOPS and 0.002 per cent in D-erythro-DOPS. L-5-HTP (Sigma, St Louis, MO), D-5-HTP (Sigma), L-DOPA (Sankyo, Tokyo, Japan), D-DOPA (Sigma), L-tryptophan (Sigma), D-tryptophan (Sigma), pargyline (Abbott, North Chicago, IL) and PALP (Sigma) were used. Other chemicals used were of reagent grade and were used without further purification.

RESULTS

Pargyline was added to the incubation mixture to protect the product formed on decarboxylation of threo-DOPS from being destroyed by monoamine oxidase. The maximum amount of NA was recovered in the presence of pargyline at the concentration of 10–100 μM. In the following experiments when 20 μM pargyline was added to the incubation medium, recovery of 1 μg NA incubated at 37° for 20 min in the incubation medium without L-threo-DOPS was 100 ± 2 per cent of the internal NA standard (1 μg) without incubation.

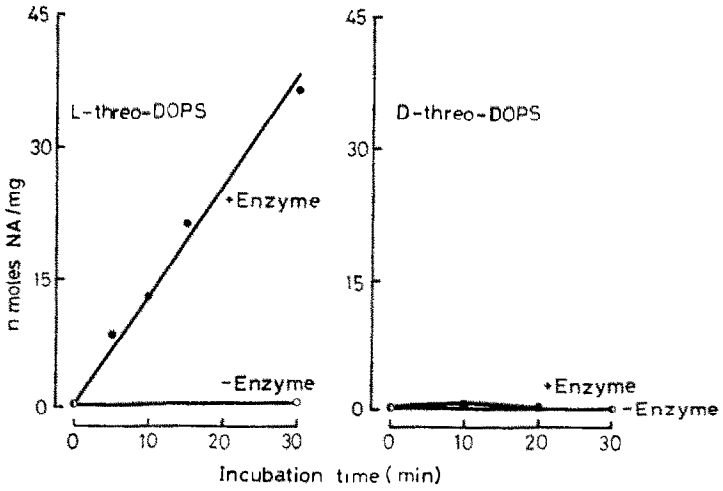

Fig. 1. Time course of decarboxylation of L- and D-threo-DOPS in the presence (+enzyme) and absence (−enzyme) of hog renal L-aromatic amino acid decarboxylase. Reactions were carried out in the medium containing 125 mM Tris–HCl (pH 8.6), 20 μM pargyline, 100 μM pyridoxal phosphate, 0.5 mM L- or D-threo-DOPS and 1.0 mg of enzyme protein at 37°. Each point represents the mean of two determinations.

Time course of decarboxylation. Production of NA from L-threo-DOPS using hog renal L-aromatic amino acid decarboxylase proceeded linearly for 30 min at 37° (Fig. 1). Decarboxylation was not observed with another optical isomer, D-threo-DOPS. Decarboxylation in the incubation medium without the enzyme was not detectable even after 30 min of incubation.

Enzyme concentration. The rate of decarboxylation of L-threo-DOPS was accelerated in proportion to the amount of enzyme in the reaction mixture and the range was 0 to 1.5 mg protein (Fig. 2A).

Temperature dependency. Incubation was carried out at 0, 10, 20, 30, 34, 37, 40 and 45° (Fig. 2B). The reaction product, NA, was not detectable after incubation at 0° and a sharp increase was noted when the incubation temperature rose to 34°. The maximum rate of decarboxylation was observed at 37° and the rate of reaction was reduced at higher temperatures (40 and 45°).

pH dependency. The incubation medium was buffered with potassium phosphate buffer (pH 7.0, 7.8 and 8.2) and Tris–HCl (pH 8.2, 8.6, 9.0 and 9.4) (Fig. 2C). Enzymic decarboxylation of L-threo-DOPS was strictly dependent on pH of the incubation medium and a maximum rate was obtained at pH 8.6.

Pyridoxal phosphate requirement. Decarboxylation of L-threo-DOPS required PALP as a cofactor. Cofactor dependency in the decarboxylation was

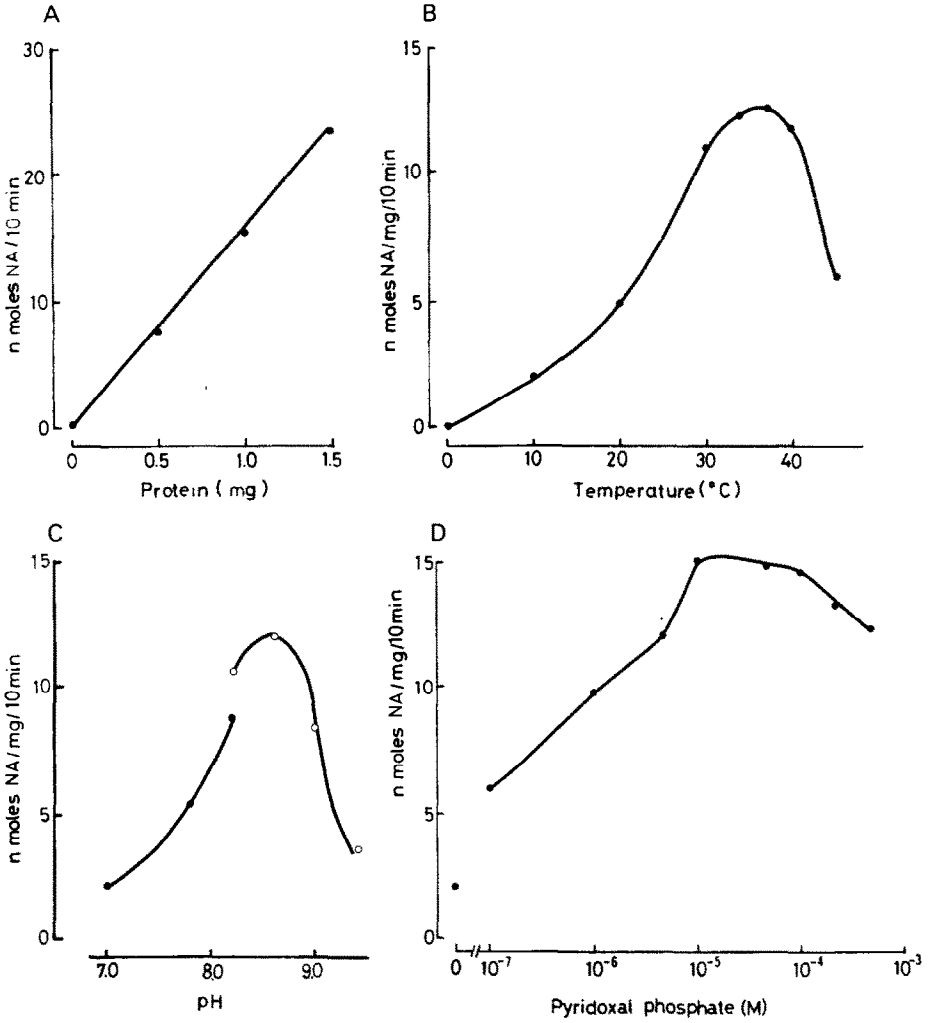

Fig. 2. Influence of concentration of enzyme protein (A), temperature (B), pH (C) and pyridoxal phosphate (PALP) (D) on the amount of noradrenaline (nmoles) formed from 0.5 mM L-threo-DOPS. (A) Reactions were carried out in the medium containing 125 mM Tris–HCl (pH 8.6), 20 μM pargyline, 100 μM PALP and 0.5 mM L-threo-DOPS in the absence or presence of enzyme protein (0.5 to 1.5 mg), at 37° for 10 min. (B) Reactions were carried out in the medium containing 125 mM Tris–HCl (pH 8.6), 20 μM pargyline, 100 μM PALP, 0.5 mM L-threo-DOPS and 1.0 mg of enzyme protein at the indicated temperature (0–45°) for 10 min. (C) Reactions were carried out in the medium containing 125 mM phosphate buffer (pH 7.0, 7.8 or 8.2) or Tris–HCl (pH 8.2, 8.6, 9.0 or 9.4), 20 μM pargyline, 100 μM PALP, 0.5 mM L-threo-DOPS and 1.0 mg of enzyme protein at 37° for 10 min. (D) Reactions were carried out in the medium containing 125 mM Tris–HCl (pH 8.6), 20 μM pargyline, 0.5 mM L-threo-DOPS and 1.0 mg of enzyme protein in the absence or presence of PALP (10^{-7} to 5×10^{-4} M) at 37° for 10 min. Each point represents the mean of two determinations.

examined over the range of 0.1–500 μM PALP (Fig. 2D). Without PALP, the rate of decarboxylation was 2.1 nmoles NA/mg of protein/10 min. The rate of decarboxylation was accelerated about 7-fold by addition of PALP, with the maximum rate at 10 μM PALP. Over 100 μM, the rate of reaction was reduced with increase in concentration of the cofactor.

K_m and V_{max} for L-threo-DOPS. Under the optimal condition for L-threo-DOPS decarboxylation, we then determined the initial velocities of the decarboxylation at 0.25–2.0 mM of the substrate concentration. From Lineweaver–Burk plots of the data, the Michaelis constant (K_m) was 4×10^{-4} M and the maximum velocity (V_{max}) was 2.80 ± 0.13 nmoles NA/mg of protein/min. For comparison, L-5-HTP decarboxylase activity of the same enzyme was measured, and K_m and V_{max} for L-5-HTP were 1.1×10^{-4} M and 5.65 nmoles 5-HT/mg of protein/min respectively.

Effects of L-isomers of other amino acids. When L-DOPA, L-5-HTP and L-tryptophan were added to the system of L-threo-DOPS decarboxylation, L-DOPA and L-5-HTP at the concentration of 0.1 mM markedly inhibited the production of NA from L-threo-DOPS, while L-tryptophan at the same concentration had no effect whatever (Fig. 3). Double reciprocal plots of the initial rates of decarboxylation of L-threo-DOPS and the concentration of L-threo-DOPS (Lineweaver–Burk plots) in the presence and absence of L-DOPA or L-5-HTP indicated that the inhibition was competitive concerning L-threo-DPS.

Effects of D-isomers of DOPS and other amino acids. D-Threo-DOPS and D-DOPA at the concentration of 0.25 mM markedly inhibited the decarboxylation of L-threo-DOPS while D-erythro-DOPS, D-5-HTP and D-tryptophan at the same concentration had no effect (Fig. 4). The effects of these D-isomers of amino acids at different concentrations on the decarboxylation of 0.5 mM L-threo-DOPS are shown in Fig. 5. D-Threo-DOPS and D-DOPA at 0.125 mM reduced the rate of decarboxylation of L-threo-DOPS by 50.4 per cent as compared to the control. D-Erythro-DOPS at a higher concentration

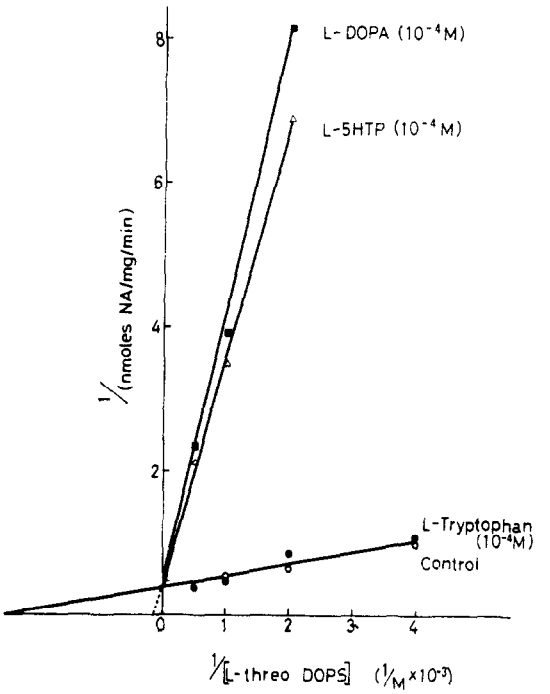

Fig. 3. Double reciprocal plots of initial rates of L-threo-DOPS decarboxylation versus concentrations of L-threo-DOPS in the presence and absence of L-isomers of other aromatic amino acids (0.1 mM). The reaction was carried out at 37° for 10 min in the presence of 125 mM Tris–HCl (pH 8.6), 20 μM pargyline, 10 μM pyridoxal phosphate, different concentrations of L-threo-DOPS and 1.0 mg of enzyme protein. Initial rates were estimated from the linear time courses of decarboxylation of L-threo-DOPS at the indicated concentrations. Each point represents the mean of two determinations.

(1 mM) slightly reduced the rate of decarboxylation while D-5HTP and D-tryptophan in concentrations up to 1 mM had no effect.

Kinetics of inhibition by D-threo-DOPS. The rate of decarboxylation of L-threo-DOPS at various concentrations was measured in the presence and absence of D-threo-DOPS (Fig. 6). Decarboxylation

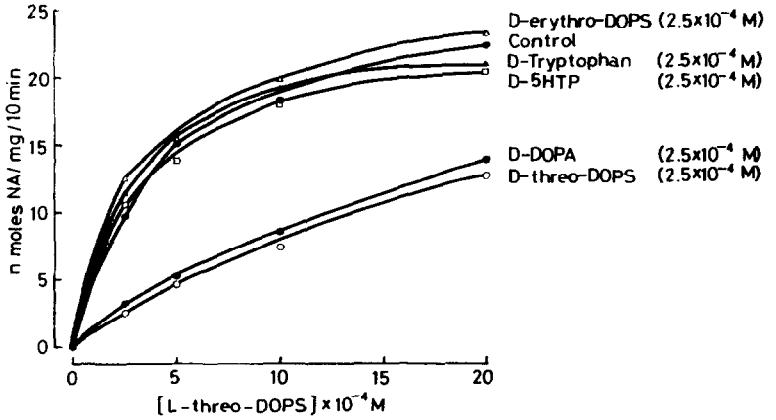

Fig. 4. Effects of D-isomers of DOPS and other aromatic amino acids (0.25 mM) on decarboxylation of various concentrations of L-threo-DOPS. The reaction was carried out at 37° for 10 min in the presence of 125 mM Tris–HCl (pH 8.6), 20 μM pargyline, 10 μM pyridoxal phosphate, different concentrations of L-threo-DOPS and 1.0 mg enzyme protein in the presence or absence of 0.25 mM D-amino acids. Each point represents the mean of two determinations.

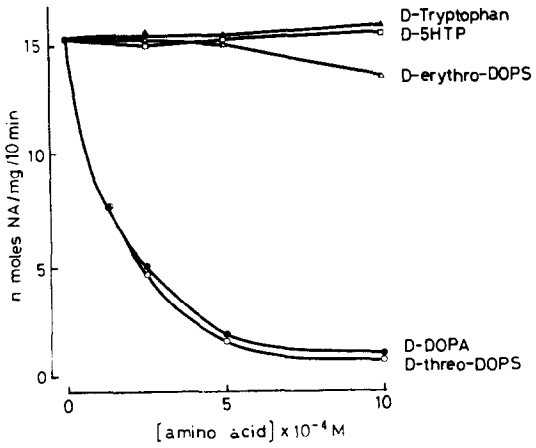

Fig. 5. Effects of D-isomers of DOPS and other aromatic amino acids up to 1.0 mM on decarboxylation of 0.5 mM L-threo-DOPS. The reaction was carried out at 37° for 10 min in the presence of 125 mM Tris–HCl (pH 8.6), 20 μM pargyline, 10 μM pyridoxal phosphate, 0.5 mM L-threo-DOPS and 1.0 mg of enzyme protein in the presence or absence of D-isomers at the indicated concentrations. Each point represents the mean of two determinations.

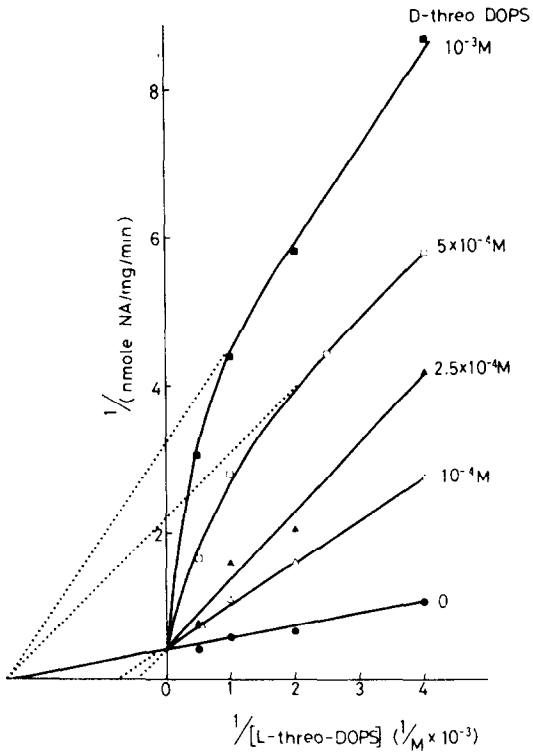

Fig. 6. Double reciprocal plots of initial rates of L-threo-DOPS decarboxylation versus concentrations of L-threo-DOPS in the presence and absence of D-threo-DOPS (0.1 to 1.0 mM). The reaction was carried out at 37° in the presence of 125 mM Tris–HCl (pH 8.6), 20 μM pargyline, 10 μM pyridoxal phosphate, L- and D-threo-DOPS at the indicated concentrations and 1.0 mg of enzyme protein. Initial rates were estimated from the linear time courses of decarboxylation of L-threo-DOPS. Each point represents the mean of two determinations.

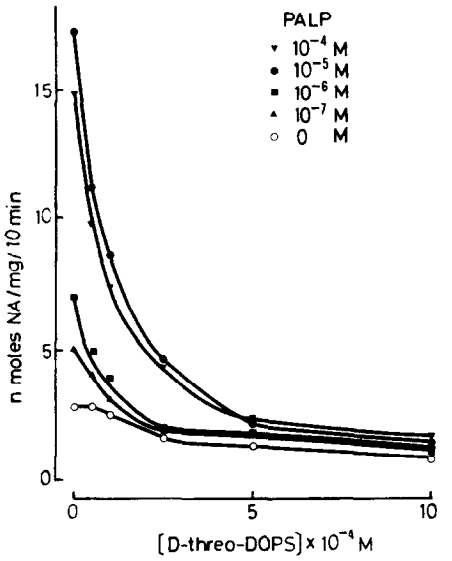

Fig. 7. Inhibitory effect of D-threo-DOPS on decarboxylation of 0.5 mM L-threo-DOPS at various concentrations of pyridoxal phosphate. The reaction was carried out at 37° for 10 min in the presence of 125 mM Tris–HCl (pH 8.6), 20 μM pargyline, 0.5 mM L-threo-DOPS, different concentrations of D-threo-DOPS and 1.0 mg of enzyme protein. Pyridoxal phosphate at the indicated concentration was added. Each point represents the mean of two determinations.

of L-threo-DOPS was competitively inhibited by D-threo-DOPS in concentrations less than 0.25 mM. At higher concentrations of D-threo-DOPS (0.5 to 1.0 mM), a noncompetitive type of inhibition was observed in the range where the concentration of L-threo-DOPS was lower than that of D-threo-DOPS. The inhibitor constant (K_i) was calculated from the points of intersection with the baseline [$= -/K_p$, $Ki = i/(K_p/Km - 1]$ as 1.3×10^{-4} M in the competitive inhibition and, from the points of the intersection with the vertical axis [$= 1/V_p$, $Ki = i/(V/V_p - 1)$], as 4.3×10^{-4} M in the noncompetitive inhibition by D-threo-DOPS.

Effect of PALP on the inhibition by D-threo-DOPS. The inhibitory effect of D-threo-DOPS on L-threo-DOPS decarboxylation was examined at various concentrations of PALP (Fig. 7). When PALP was not added to the incubation medium, decarboxylation of L-threo-DOPS was inhibited by 0.5 mM D-threo-DOPS to 45 per cent that of the control. Decarboxylation which was accelerated by exogenously added PALP was more sensitively inhibited by D-threo-DOPS. Fifty per cent inhibition of the decarboxylation accelerated by PALP (0.1 μM to 0.1 mM) was observed at 0.05–0.1 mM of D-threo-DOPS. The inhibition by D-threo-DOPS was not altered even with an excess addition of PALP (0.1 mM).

DISCUSSION

Formation of NA occurred after incubation of the enzyme with L-threo-DOPS but not with D-threo-DOPS, indicating that the enzyme preparation used in this study did not include the D-amino acid oxidase-L-amino-transferase system, which has

been reported by Shindo *et al.* [12] to be localized in the rat kidney and to convert D-amino acid to its L-isomer. In addition, decarboxylation of threo-DOPS by L-aromatic amino acid decarboxylase is apparently stereospecific.

The maximum rate of L-threo-DOPS decarboxylation was observed at pH 8.6. This optimal pH was similar to that required for other substrates, except L-DOPA [9, 11]. Decarboxylation of L-DOPA is usually carried out at neutral pH because of its instability at alkaline pH. Despite a configuration similar to that of L-DOPA, L-threo-DOPS is relatively stable in an alkaline solution.

Decarboxylation of L-threo-DOPS was competitively inhibited by L-DOPA and L-5-HTP, suggesting that a single enzyme catalyzes decarboxylation of L-threo-DOPS, L-DOPA and L-5-HTP. L-Tryptophan did not inhibit the decarboxylation of L-threo-DOPS, probably due to the low affinity of this amino acid to the enzyme.

From the K_m values, the affinity of L-threo-DOPS to the enzyme is considered to be slightly lower than that of L-5-HTP. The maximum velocity (V_{max}) of decarboxylation of L-threo-DOPS was about one half that of L-5-HTP. Porter *et al.* [4] reported that racemic threo-DOPS was decarboxylated by the high speed supernatant (30,000 *g* 30 min) of rat kidney homogenate at about 0.01 the rate of 5-HTP at pH 8.0. Assuming that one half of the racemic compound exists as L-isomer, this rate of threo-DOPS decarboxylation is far slower than that expected on the basis of the present experiments with purified hog kidney decarboxylase. This discrepancy between the velocity of decarboxylation of L- and racemic threo-DOPS may to some extent be explained by the pH of the incubation medium in their experiments which, although adequate for 5-HTP decarboxylation, is not so for L-threo-DOPS decarboxylation. However, a more likely reason is the inhibitory effect of D-threo-DOPS on L-threo-DOPS decarboxylation. In our previously reported work we used a supernatant fraction of rat kidney homogenate centrifuged at 8000 *g* for 10 min and found that the rate of decarboxylation of racemic threo-DOPS was far below one half that of L-threo-DOPS, and when D-threo-DOPS was added to the incubation medium, the rate of decarboxylation of L-threo-DOPS was reduced [7]. Using partially purified L-aromatic amino acid decarboxylase of hog kidney, we confirmed herein the inhibition of L-threo-DOPS decarboxylation by D-threo-DOPS. Furthermore, the inhibitory effect of D-DOPA was as potent as that seen with D-threo-DOPS. The inhibition of L-threo-DOPS decarboxylation by these D-isomers is probably not due to the inactivation of PALP as an excess amount of PALP did not reverse the inhibitory effect.

With D-erythro-DOPS, a higher concentration was required for manifestation of the inhibitory effect and this effect was not observed with D-isomers of 5HTP and tryptophan. It is interesting to consider that inhibition of the decarboxylation by D-aromatic amino acids is due to 2R configuration in their molecules, and that 1R configuration in D-erythro-DOPS is responsible for the low affinity of this compound to the enzyme.

Various α-alkylated aromatic amino acids such as α-methyl-DOPA reportedly inhibit L-aromatic amino acid decarboxylase activity [13]. Among these compounds, only L-isomers have been considered to be potent [14, 15]. These α-alkylated amino acids are not only inhibitors but also substrates of the enzyme, and inhibition is reversible by the addition of PALP [16, 17]. Thus, inhibition by D-threo-DOPS and D-DOPA is distinguishable from that by α-alkylated aromatic amino acids.

A D-aromatic amino acid orally given to rats shows a higher accumulation and longer retention in several tissues including brain and kidney, as compared with its L-isomer [18]. Thus, the D-amino acid may interact *in vivo* with L-aromatic amino acid decarboxylase, and the purified L-threo-DOPS may act as a more effective precursor of natural NA as compared with racemic threo-DOPS.

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