DOPA AND 5-HTP DECARBOXYLASE(S) IN BRAIN

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BY

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A thesis submitted to the Faculty of Graduate Studies of
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DOCTOR OF PHILOSOPHY

To my parents, grandpa,

and my sister

They must often change who would

be constant in happiness and wisdom

Confucius

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ABSTRACT

Aromatic L-amino acid decarboxylase (AADC, EC 4.1.1.28) is a pyridoxal phosphate-dependent enzyme and has been generally regarded to be a single protein entity capable of catalyzing the decarboxylation of both 3,4-dihydroxyphenylalanine (DOPA) to dopamine and 5-hydroxytryptophan (5-HTP) to serotonin. The presence of more than one decarboxylase for aromatic amino acids in different organs and brain regions has also been suggested by various lines of evidence including non-parallel changes in brain serotonin and catecholamine levels in pyridoxine-deficient rats. The present study was designed to test the hypothesis that the decarboxylation of DOPA and 5-HTP are regulated independently. The objective also included the isolation and characterization of the neuronal DOPA decarboxylase

Pyridoxine deficiency has been shown to have differential effects on the activity of rat brain AADC using either DOPA or 5-HTP as substrate. Regional differences in the distribution of AADC activity towards DOPA and 5-HTP in the rat and bovine brain have also been shown. Pyridoxal phosphate also binds more tightly to DOPA decarboxylase than to 5-HTP decarboxylase. Furthermore, the neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and its metabolite, MPP^+, have been shown to stimulate 5-HTP decarboxylase but not DOPA decarboxylase. Dissimilar responses of DOPA decarboxylase and 5-HTP decarboxylase to α-methyl DOPA, aminooxyacetic acid, hydralazine, and phenelzine have been demonstrated as well.

The above results are consistent with the suggestion that these two decarboxylations are catalyzed by separate proteins. Conclusive evidence for this notion could only come from the purification of the suggested proteins. DOPA decarboxylase has now been purified from the bovine striatum (neuronal) and has properties, such as optimum pH, molecular weight, sulfhydryl group requirement, and inhibition by heavy metal ions, similar to the non-neuronal (e.g. kidney, adrenal) enzyme. However, the neuronal enzyme differs from the non-neuronal enzyme in its heat lability and sensitivity to inhibitors such as aminooxyacetic acid.

The results of the present study suggest that the decarboxylation of DOPA and 5-HTP are regulated independently. However, the resolution of the question whether one or multiple proteins are involved in the decarboxylations of DOPA and 5-HTP in mammalian neuronal tissues will have to await the purification and characterization of 5-HTP decarboxylase as well.

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ABBREVIATIONS

5-HT:	Serotonin (5-Hydroxytryptamine)
5-HTP:	5-Hydroxytryptophan
AADC:	Aromatic L-amino acid decarboxylase
AOAA:	Aminooxyacetic acid
CAD/CSAD:	Cysteic acid/cysteinesulfinic acid decarboxylase
CNS:	Central nervous system
CO_2:	Carbon dioxide
DA:	Dopamine
DFMD:	D,L-α-Difluoromethyl-DOPA
DOPA:	3,4-Dihydroxyphenylalanine
DTT:	DL-Dithiothreitol
EEG:	electroencephalogram
GABA-T:	GABA-transaminase
GABA:	γ-Aminobutyric acid
GAD:	Glutamic acid decarboxylase
HDC:	Histidine decarboxylase
HPLC:	High performance liquid chromatography
MAO:	Monoamine oxidase
MFMD:	D,L-α-Monofluoromethyl-DOPA
$MPDP^+$:	1-methyl-4-phenyl-2,3-dihydropyridinium cation
MPP^+:	1-Methyl-4-phenylpyridinium
MPTP:	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NAS:	N-Acetylserotonin
NSD 1015:	*m*-Hydroxybenzylhydrazine

NSD 1034: N-(3-Hydroxybenzyl)-N-methylhydrazine dihydrogen phosphate

NSD 1055: 4-Bromo-3-hydroxybenzyloxyamine dihydrogen phosphate

PAGE: Polyacrylamide gel electrophoresis

PLP: Pyridoxal 5'-phosphate

SDS: Sodium dodecyl sulfate

CHAPTER I

INTRODUCTION

Introduction

Pyridoxine-dependent enzymes have been widely investigated in the central nervous system. The synthesis of the neurotransmitters, dopamine, norepinephrine, and serotonin, involves a pyridoxal phosphate (PLP)-dependent decarboxylation step. It has been suggested that the enzyme involved, aromatic L-amino acid decarboxylase is a single protein entity capable of decarboxylating a variety of amino acids including 3,4-dihydroxyphenylalanine (DOPA) and 5-hydroxytryptophan (5-HTP). In view of the contradictory evidence, there is as yet no agreement on the unitary nature of this enzyme. The presence of more than one decarboxylase for aromatic amino acids in different organs or brain regions has been suggested by various lines of evidence. This includes earlier work from this laboratory which reported that pyridoxine deficiency causes non-parallel changes in the brain serotonin and catecholamine levels. The affinity of the coenzyme for the different apodecarboxylases could vary. The activity of the decarboxylase with the most tightly bound coenzyme would be higher than those with lesser affinities between the apoenzyme and coenzyme. The objective of this thesis is to investigate the enzymes responsible for the decarboxylation of DOPA and 5-HTP in the mammalian brain. The results of this study would enable us to examine the regulation of these enzymes.

The thesis is divided into three major chapters: a literature review section, an experimental section and a general discussion. The literature review begins with a brief discussion on vitamin B_6 and its role in PLP-dependent reactions. This is followed by a review of many

aspects of the enzyme of interest, aromatic L-amino acid decarboxylase (AADC); these includes the tissue distribution, methods of assay, purification, and some general properties of the enzyme. Following that is a review of the various lines of evidence in support of the "single enzyme" and "separate enzyme" hypotheses. The final part of the literature review deals with certain features of the experimental pyridoxine deficiency, with special emphasis on the changes in brain monoamine levels and the physiological consequences of decreased brain serotonin levels, resulting from pyridoxine deficiency.

The experimental results are divided into two sections with a brief introduction and discussion for the individual sections therein. The first section is divided into two parts which characterize the nature and properties of the decarboxylation of DOPA and 5-HTP by AADC in the rat brain. Part A delineates the effects of pyridoxine deficiency on AADC while part B describes the *in vitro* effects of the neurotoxins, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, and 1-methyl-4-phenylpyridinium on AADC. Also included in this latter part is a brief examination of the effects of various decarboxylase inhibitors and anti-pyridoxine metabolites on AADC. The second section deals with the purification and characterization of DOPA decarboxylase from bovine striatum.

A general discussion section is presented in the last chapter.

CHAPTER II

LITERATURE REVIEW

LITERATURE REVIEW

A. Vitamin B_6: An Overview

1. General Metabolism

Vitamin B_6 is a generic descriptor which includes three closely related compounds, pyridoxine, pyridoxal, and pyridoxamine, which are interconvertible biologically (Dakshinamurti, 1977; Ebadi and Govitrapong, 1980; Dakshinamurti, 1982; Lehninger, 1982; Bender, 1985). All three forms of the vitamin can also exist as the 5'-phosphate esters, pyridoxine 5'-phosphate, pyridoxal 5'-phosphate (PLP), and pyridoxamine 5'-phosphate. The interconversions of the B_6 vitamers are shown in Figure 1. All three non-phosphorylated vitamers are phosphorylated by pyridoxal kinase [EC 2.7.1.35; also known as pyridoxine kinase and pyridoxamine kinase], an enzyme which is found in a variety of tissues (McCormick and Merill, 1980). In the brain, pyridoxine kinase is regulated by several factors such as inhibition of enzyme activity by the levels of PLP and monoamines, and activation by brain phospholipids (Dakshinamurti, 1977,1982). Pyridoxine and pyridoxine 5'-phosphate are acted upon by pyridoxine 4-oxidase [EC 1.1.3.12] to yield the aldehydes (Bender, 1985). This oxidase is strongly inhibited by PLP, the end-product of the pathway (Dakshinamurti, 1977,1982; Bender, 1985). Thus, the kinase and oxidase could control the cellular level of PLP. In the free form, the phosphorylated coenzymes are hydrolyzed by the various phosphatases. To serve as coenzymes, a large part of PLP is protein-bound. Thus, the

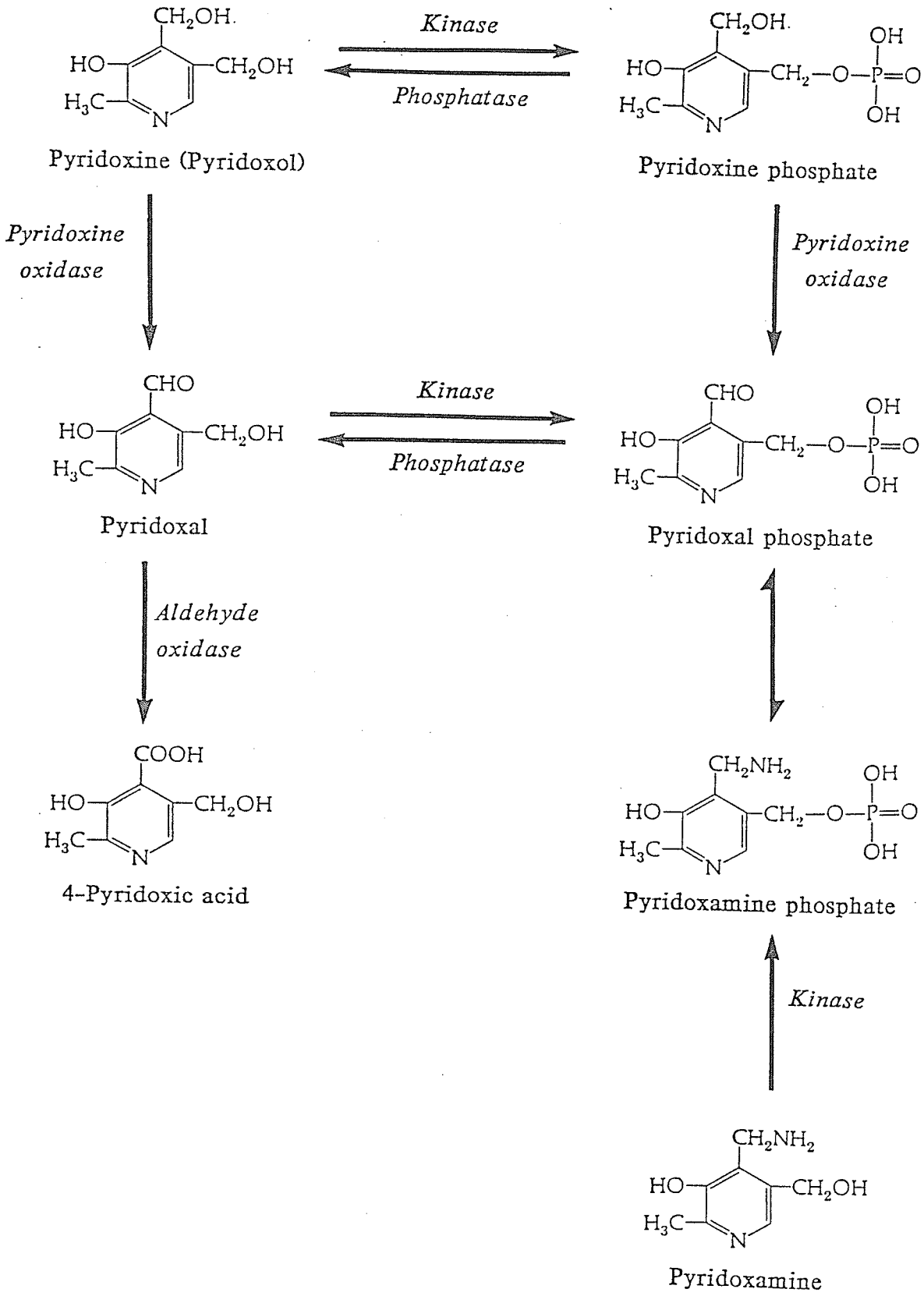

Figure 1. The metabolism of vitamin B_6

levels of PLP in the tissue is balanced by the rates of its synthesis, sequestration as the protein-bound form and by the rate of its degradation by phosphatases (Dakshinamurti, 1977,1982). Any excess of the vitamin B_6 is rapidly metabolized to 4-pyridoxic acid (Bender, 1985).

2. Biochemical Role as a Coenzyme

Pyridoxal phosphate is the major coenzymatic form of vitamin B_6 although pyridoxamine phosphate can also function as a coenzyme in transamination reactions (Dakshinamurti, 1977,1982; Bender, 1985). The principal biochemical reaction is between the carbonyl group of PLP with a primary amine (usually a lysine residue of a protein) to form a Schiff base (Figure 2). The 5'-phosphate group of PLP is required for binding to ensure the correct alignment of the coenzyme at the catalytic site of the enzyme (Bender, 1985). In the presence of a substrate, the internal Schiff base is displaced by the formation of a Schiff base aldimine via the condensation between the carbonyl group of PLP and the -amino group of the substrate (Figure 3). This results in a destabilized carbon atom of the substrate that is adjacent to the aldimino group. Many further reactions can take place depending on the site of elimination and replacement of the substituents. These reactions include deamination, decarboxylation, desulfination, oxidative deamination, racemization, and transamination (Dakshinamurti, 1982; Bender, 1985). The mechanism for PLP-dependent decarboxylation has also been proposed (Boeker and Snell, 1972; Snell, 1981).

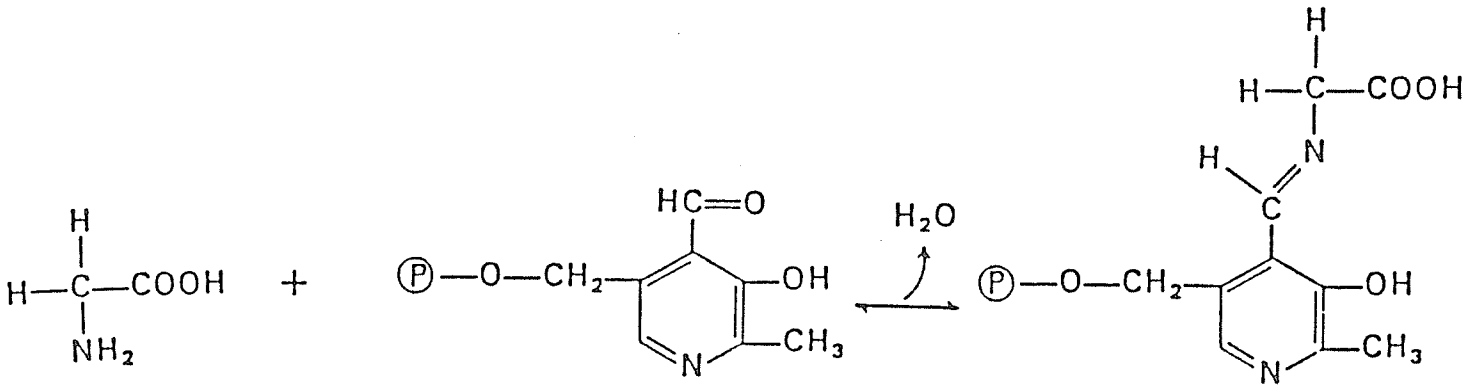

Figure 2. Formation of Schiff base between pyridoxal phosphate and a primary amine.

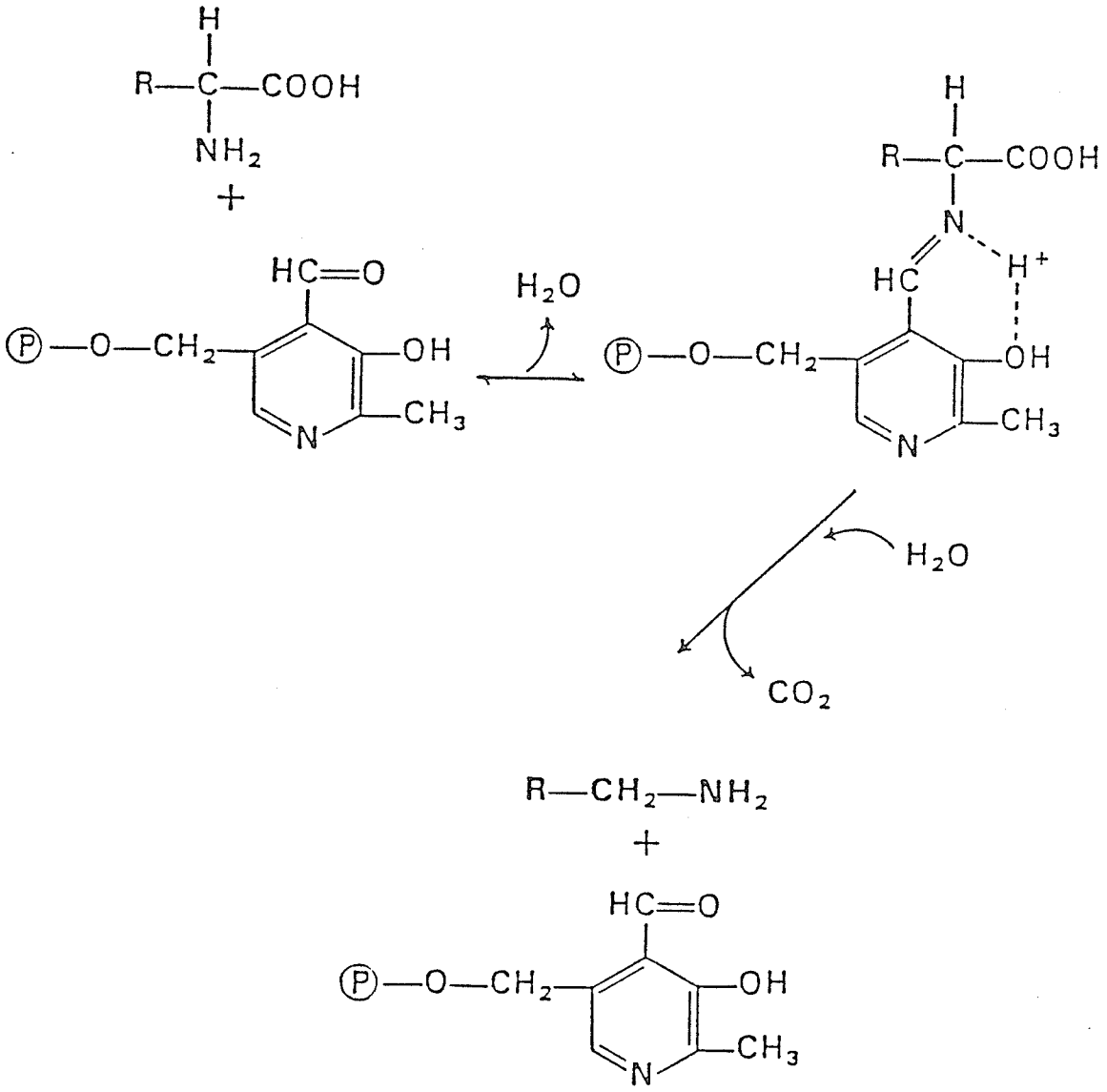

Figure 3. The reaction of pyridoxal phosphate with amino acid substrate in α-decarboxylation (Bender, 1985).

As a result of the destabilization of the substrate, a series of reactions can take place. These can be classified into three groups depending on which carbon atom of the substrate is involved (Dakshinamurti, 1977,1982). Reactions occurring at the α-carbon atom are catalyzed by transaminases [EC 2.6.1.x], racemases of α-amino acid [EC 5.1.1.x], amino acid α-decarboxylases [EC 4.1.1.x], and enzymes catalyzing condensation of glycine and the α-β cleavage of β-hydroxy amino acids such as 5-aminolevulinic acid synthetase [EC 2.3.1.37], glycine hydroxymethylase [EC 2.1.2.1; serine hydroxymethylase], and serine palmitoyltransferase [EC 2.3.1.50]. The reactions that occur at the β-carbon atom are catalyzed by serine dehydratase [EC 4.2.1.13] threonine dehydratase [EC 4.2.1.16], cystathionine β-synthase [EC 4.2.1.22], tryptophan synthase [EC 4.2.1.20], tryptophanase [EC 4.1.99.1], kynureninase [EC 3.7.1.3], and aspartate 4-decarboxylase [EC 4.1.1.12]. The last group of reactions which occur at the γ-carbon atom of the substrate are catalyzed by enzymes such as homocysteine desulfhydrase [EC 4.4.1.2] and γ-cystathionase [EC 4.4.1.1]. Besides this, a structural role for PLP in glycogen phosphorylase [EC 2.4.1.1] has also been demonstrated (Hedrick, 1972). In the brain, energy is supplied mainly by blood glucose rather than from glycogen as the glycogen reserves are modest. Thus, in pyridoxine deficiency, the decrease in phosphorylase activity did not have any adverse effect on cerebral glucose metabolism (Dakshinamurti, 1982).

3. Role of Vitamin B_6 Enzymes in the Nervous System

Pyridoxal phosphate-dependent enzymes have been widely investigated in the nervous system (Dakshinamurti, 1977,1982; Dakshinamurti *et al.*, 1985a). These enzymes include the various amino acid decarboxylases which are involved in the synthesis of various diamines and polyamines (putrescine, spermidine, and spermine), and various putative neurotransmitters such as dopamine, serotonin, tyramine, tryptamine, histamine, γ-aminobutyric acid (GABA), and taurine (Figure 4). Therefore, vitamin B_6 plays a crucial role in maintaining an equilibrium of neurotransmitters, via the regulation of their syntheses, in the nervous system.

The synthesis of all sphingolipids - sphingomyelin, cerebrosides, sulfatide, globosides, and gangliosides - stems from the initial synthesis of sphingosine. Sphingosine, in turn, is derived from the condensation of serine and palmitoyl CoA, a reaction catalyzed by a PLP-dependent enzyme: serine palmitoyltransferase. In pyridoxine deficiency, sphingolipids synthesis is impaired leading to defective myelination seen in experimental animals (Dakshinamurti, 1982). The involvement of vitamin B_6 in polyamine synthesis also plays a crucial role during the maturation period of the nervous system. Since PLP functions as a cofactor for ornithine decarboxylase, pyridoxine deficiency will lead to a decrease in enzyme activity and polyamine synthesis (Dakshinamurti, 1982). This may affect protein synthesis through effects on gene expression by polyamines. A defective synthesis of various neuronal proteins - both structural and functional - in

AROMATIC AMINO ACID DECARBOXYLASE

3,4-Dihydroxyphenylalanine ⟶ Dopamine + CO_2

5-Hydroxytryptophan ⟶ Serotonin + CO_2

Phenylalanine ⟶ Phenylethylamine + CO_2

Tyrosine ⟶ Tyramine + CO_2

CYSTEIC ACID DECARBOXYLASE

Cysteic acid ⟶ Taurine + CO_2

GLUTAMIC ACID DECARBOXYLASE

Glutamic acid ⟶ γ-Aminobutyric acid + CO_2

HISTIDINE DECARBOXYLASE

Histidine ⟶ Histamine + CO_2

ORNITHINE DECARBOXYLASE

Ornithine ⟶ Putrescine + CO_2

Figure 4. Pyridoxal 5'-Phosphate dependent amino acid decarboxylases

combination with impaired myelination will have drastic effects in the functional integrity of the central nervous system.

B. Aromatic L-Amino Acid Decarboxylase (EC 4.1.1.28)

1. Distribution

The PLP-dependent enzyme, aromatic L-amino acid decarboxylase (AADC) has been suggested to lack substrate specificity and to be involved in the synthesis of the neurotransmitters, dopamine and serotonin (Lovenberg *et al.*, 1962) (Figure 5). Since dopamine is a precursor for norepinephrine synthesis, AADC also plays a role here. However, the decarboxylation step is not generally considered to be the rate-limiting step in the biosynthesis of dopamine, norepinephrine, or serotonin. The hydroxylation of the precursor amino acids is considered to be the rate-limiting step (Figure 5). Aromatic L-amino acid decarboxylase has been studied extensively in a variety of mammalian tissues especially the adrenals, brain, kidney, and liver (Table 1) (Boeker and Snell, 1972; Sourkes, 1979, 1987; Ebadi and Govitrapong, 1980). In the brain, the concentration of the enzyme is very low and there is considerable regional variation in the distribution of the enzyme activity (Sourkes, 1979). This was observed in both the human brain (Lloyd and Hornykiewicz, 1972) and the rat brain (Sims *et al.*, 1973) (Table 2).

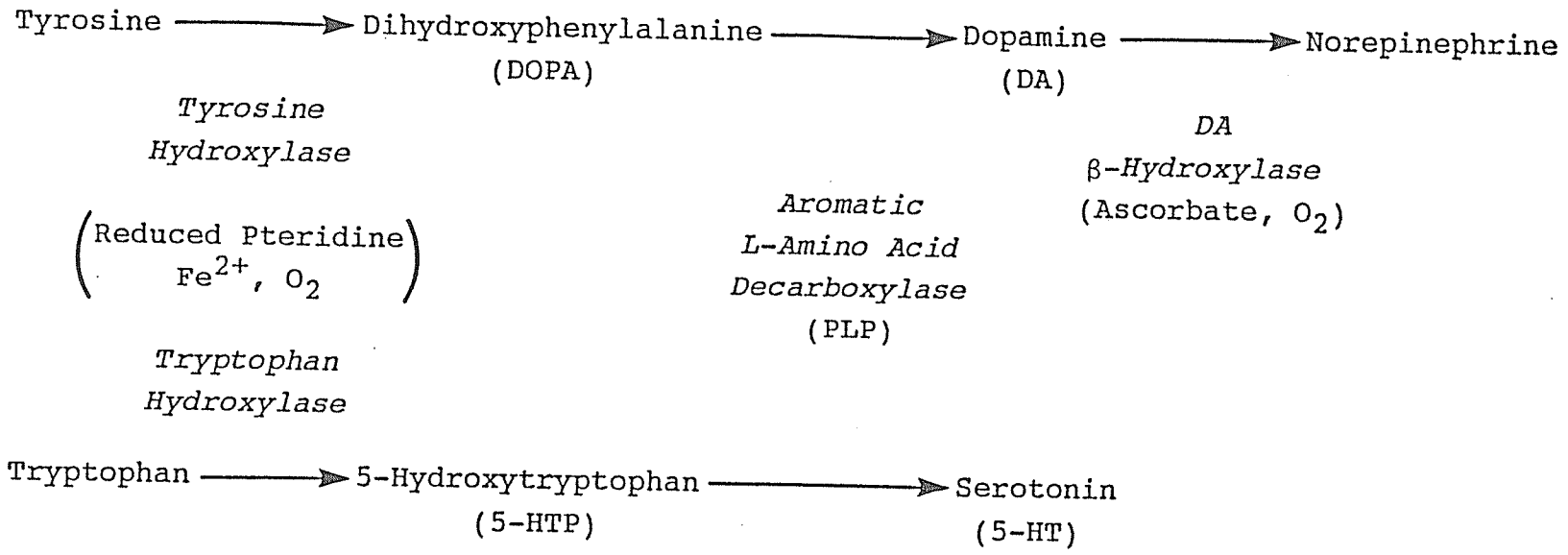

Figure 5. The Biosynthetic Pathways of Catecholamines and Serotonin.

Table 1

Distribution of Aromatic L-Amino Acid Decarboxylase in Mammalian Tissues

Tissue Source	DOPA decarboxylase	5-HTP Decarboxylase
Rat		
Adrenals	353[b]	44[b]
Brain	167[a]	50[a]
Heart	12[b]	2[b]
Kidney	2300[a]	433[a]
Large Intestine	45[b]	10[b]
Liver	1867[a]	400[a]
Lung	117[a]	17[a]
Pineal	1400[b]	277[b]
Serum	0.060[c]	0.034[c]
Small Intestine	850[a]	350[a]
Spleen	5[b]	1[b]
Guinea Pig		
Adrenals	50[a]	50[a]
Brain	83[a]	50[a]
Heart	33[a]	33[a]
Kidney	2633[a]	1167[a]
Liver	717[a]	200[a]
Lung	17[a]	17[a]
Serum	0.35[c]	0.18[c]
Small Intestine	1217[a]	867[a]
Spleen	17[a]	17[a]

[a]Values are expressed in nmoles of product formed (dopamine or serotonin)/min/g tissue dry wt. (Davis and Awapara, 1960)

[b]Values are expressed in nmoles of product formed (dopamine or serotonin)/min/g tissue wet wt. (Rahman *et al.*, 1981b).

[c]Values are expressed in nmoles of product formed (dopamine or serotonin)/min/ml (Rahman *et al.*, 1981a).

Table 2

Regional distribution of DOPA Decarboxylase in the Human Brain and the Rat Brain

Brain Regions	Human Brain[a]	Rat Brain[b]
Amygdala	28	- [c]
Caudate nucleus	366	-
Corpus striatum	-	18.7
Cerebellum	28	1.6
Dorsal raphe	134	-
Frontal cortex	32	3.1
Hippocampus	n.d.	2.4
Hypothalamus	149	8.6
Locus caeruleus	46	-
Medulla oblangata (whole)	24	-
Medulla-pons, Lateral	-	3.8
Medulla-pons, Midline	-	5.4
Mesencephalic tegmentum	252	9.0
Midbrain tectum	77	5.2
Occipital cortex	22	1.9
Parietal cortex	21	-
Pineal gland	213	-
Putamen	432	-
Thalamus	21	-

[a]Values are enzyme activity expressed as nmoles of CO_2 formed/2 h/100 mg protein; n.d.= not detectable (Lloyd and Hornykiewicz, 1972).

[b]Values are enzyme activity expressed as umoles of CO_2 formed/h/g wet weight of tissue (Sims *et al.*, 1973).

[c]This activity was not determined.

2. Assay Methods

Aromatic L-amino acid decarboxylase can be assayed by a variety of methods of differing sensitivities and levels of simplicity (Sourkes and Lancaster, 1972; Duncan and Sourkes, 1978; Culvenor and Lovenberg, 1983; Sourkes, 1987). These methods measure the formation of either of the decarboxylation products, the amine (usually dopamine or serotonin) or carbon dioxide. The reaction, using either 3,4-dihydroxyphenylalanine (DOPA) or 5-hydroxytryptophan (5-HTP) as substrate, proceeds as follows:-

$$\text{DOPA} \xrightarrow{\text{PLP}} \text{Dopamine} + CO_2$$

$$\text{5-HTP} \xrightarrow{\text{PLP}} \text{Serotonin} + CO_2$$

(a) *Measurement of the amine formed*

(i) *Separation of the amine by ion-exchange chromatography* - Activated Permutit (Dietrich, 1953) and activated alumina (Anton and Sayre, 1962; Freed and Asmus, 1979) were initially used for extracting the amines formed. These were replaced by the more versatile ion exchangers, Dowex 50 (Bertler *et al.*, 1958; Hakanson and Owman, 1966) and Amberlite CG50 (Davis and Awapara, 1960; Roberge, 1977) for retaining dopamine (DA) and serotonin (5-HT), respectively. Radiolabeled substrates have also been used to increase the sensitivity of this technique (Hakanson and Owman, 1966).

(ii) *Separation of the amine by solvent partition* - This procedure involves the extraction of the product into 1-butanol directly from the

reaction mixture, which has been saturated with sodium chloride. This technique has been used to extract 5-HT at pH 10 (Kuntzman *et al.*, 1961) and has been adapted to extract DA at neutral pH (Laduron and Belpaire, 1968). Mixtures of butanol-chloroform (Snyder and Axelrod, 1964) or butanol-benzene (Christenson *et al.*, 1970) have also been used for extraction. To improve sensitivity of assays using this technique, [^{14}C]- and [^{3}H]-labeled substrates have been used.

(iii) *Separation of the amine by high performance liquid chromatography (HPLC)* - This relatively new technique offers much higher sensitivity than previous ones, especially when combined with electrochemical detection. However, this technique requires careful manipulation in order to attain high sensitivity and reproducibility. The most widely used column is the C_{18} reverse phase column (e.g. uBondapak Ultrasphere ODS or Yanapak ODS-T or CP MicroSpher). This column is used in conjunction with a mobile phase consisting of a mixture of an acidic (or neutral) aqueous buffer, sometimes with a certain proportion of methanol and/or acetonitrile. The method has been used in the determination of AADC activity using either DOPA (Nagatsu *et al.*, 1979; D'Erme *et al.*, 1980; Rahman *et al.*, 1981a,b; Rahman and Nagatsu, 1982; Boomsma *et al.*, 1986; Lee *et al.*, 1986) or 5-HTP (Rahman *et al.*, 1980, 1981a,b; Rahman and Nagatsu, 1982) as substrate. Most of these methods were used in combination with an electrochemical detector except for a few which utilize spectrofluorimetric detection of native fluorescence after excitation at 254 nm (Anderson and Purdy, 1979), spectrofluorimetric detection with pre-column derivatization (excitation at 350 nm and emission at 480 nm; Lee *et al.*, 1985, 1986), and detection of absorbance

at 280 nm (D'Erme *et al.*, 1980). The HPLC procedures have allowed the determination of AADC activity in a variety of tissues (Rahman *et al.*, 1981b; Rahman and Nagatsu, 1982; Lees *et al.*, 1985) including serum and plasma, which have very low enzyme activity (Rahman *et al.*, 1981a; Boomsma *et al.*, 1986; Lees *et al.*, 1985, 1986).

(iv) *Other separation and detection methods for the amine* - The DA formed from the decarboxylation of DOPA can be oxidized by bovine serum amine oxidase conjugated to Sepharose 4B. The aldehyde thus formed by the immobilized enzyme can then reduce NAD, in the presence of aldehyde dehydrogenase, to form NADH leading to an increase in absorbance at 340 nm (Rosei *et al*, 1984). Two other methods involve the derivatization of DA with pentafluoropropionic anhydride, followed by gas chromatography (Wong and Sandler, 1974), or the derivatization of DA with 1,2-diphenylethylenediamine, followed by direct spectrofluorimetry or by reversed phase HPLC with fluorimetric detection (Lees *et al.*, 1985). The liquid cation exchanger, bis-diethylhexylphosphoric acid in chloroform, which separates the amine products from the amino acid substrates, has also been used (McCaman, 1972; Vaccari, 1976). Saavedra (1976) also reported a sensitive radioenzymatic microassay for 5-HTP decarboxylase. This assay requires a stepwise enzymatic conversion of 5-HT to [^{3}H]melatonin via the intermediate methylation (using [^{3}H-methyl]-S-adenosyl-methionine) of N-acetylserotonin. A spectrophotometric assay for DOPA decarboxylase has also been reported (Sherald *et al.*, 1973; Charteris and John, 1975). This method requires the reaction of DA with trinitrobenzenesulfonic acid to form

trinitrophenyldopamine, which is then extracted into benzene and quantitated by measuring its absorbance at 340 nm.

(b) *Measurement of the CO_2 release*

(i) *Manometric* - This technique is only of historic significance whereby the Warburg apparatus is used (Culvenor and Lovenberg, 1983; Sourkes, 1987). However, this technique has led to the development of the radiochemical $^{14}CO_2$ trapping technique, which is now being used widely for many decarboxylase assays.

(ii) *Radiometric* - This sensitive radiochemical technique measures the release of $^{14}CO_2$ from [carboxyl-^{14}C]-labeled substrates. The reaction is allowed to proceed in an enclosed system and thus the radioactive CO_2 released from the reaction mixture is captured on alkali (KOH)-soaked filter paper or in hyamine hydroxide or in NCS (Amersham Radiochemical). This method originally was used to assay only DOPA decarboxylase activity (Christenson *et al.*, 1970; Lloyd and Hornykiewicz, 1970; Lamprecht and Coyle, 1972). With the recent availability of the [carboxyl-^{14}C]-5-HTP, this method can now be adapted to measure 5-HTP decarboxylase activity as well. A major criticism of this technique is that it also measures the non-enzymatic breakdown of DOPA, an event occurring under certain incubation conditions, both in the presence and absence of tissue (Vogel *et al.*, 1970, 1972; Mackowiak *et al.*, 1972). However, this non-enzymatic decarboxylation of DOPA can be prevented by the addition of EDTA and 2-mercaptoethanol (Mackowiak *et al.*, 1972, 1975; Okuno and Fujisawa, 1983).

It should also be noted that in the enzymatic decarboxylation of either DOPA or 5-HTP, the cofactor PLP may form adducts with either substrate (Schott and Clark, 1952; Tran, 1972; Dominici *et al.*, 1984), resulting in a decrease in enzyme activity.

3. Purification

DOPA decarboxylase has been purified to apparent homogeneity from tissues such as the guinea pig, pig, and rat kidneys (Christenson *et al.*, 1970; Lancaster and Sourkes, 1972; Srinivasan and Awapara, 1978; Borri Voltattorni *et al.*, 1979; Shirota and Fujisawa, 1988), bovine adrenals (Ceasar et al., 1970; Goldstein, 1972; Albert et al., 1987), rat liver (Awapara *et al.*, 1962; Ando-Yamamoto *et al.*, 1987; Dominici *et al.*, 1987) and human pheochromocytoma (Maneckjee and Baylin, 1983; Ichinose *et al.*, 1985) (Tables 3(a) and 3(b)). The enzyme has also been partially purified from the small intestine of monkey (Murali and Radhakrishnan, 1970). Recently, the purification of DOPA decarboxylase from bovine brain stem, by affinity chromatography using a monoclonal antibody against the adrenal enzyme, has been reported by Nishigaki and co-workers (1988).

The individual papers should be consulted for detailed purification schemes. Generally, the purification procedures involved the initial extraction of DOPA decarboxylase with isotonic sucrose or phosphate buffer, containing stabilizers such as PLP, 2-mercaptoethanol, and EDTA. This was usually followed by ammonium sulfate fractionation or acid, heat or protamine sulfate treatment (see footnotes to Tables 3(a) and 3(b)). The range of saturation most commonly used for the

TABLE 3 (a)

Purification of DOPA Decarboxylase from Pig, Guinea Pig, and Rat Kidneys

Procedure[a]	A	B	C	D	E
Tissue source	Pig kidney	Pig kidney	Pig kidney	Rat kidney	Guinea pig kidney
Assay conditions					
Substrate	[1-^{14}C]DOPA	DL-5-HTP[b]	L-DOPA	[1-^{14}C]DOPA	*o*-tyrosine[c]
Temperature	37°C	37°C	25°C[d]	37°C	37°C
pH	7.0	6.8	6.8	7.0	7.1
Specific activity[e]	8670	352	3069	11,056	9350
Purification fold	332	192	209	1024	334
Yield (%)	5.9	18	35	14	9

[a]Procedure A: Ammonium sulfate fractionation, heat treatment (6 min at 50°C), Alumina C_{gamma} gel, Polyethylene glycol precipitation, DEAE-cellulose, DEAE-Sephadex, Hydroxyapatite (Christenson *et al.*, 1970).

B: Heat treatment (5 min at 50°C), Ammonium sulfate fractionation, DEAE-Sephadex, Hydroxyapatite, Sephadex G-150 (Lancaster and Sourkes, 1972).

C: Ammonium sulfate fractionation, DEAE-cellulose, QAE-Sephadex, Sephadex G-100 superfine (Borri Voltattorni *et al.*, 1979).

D: Ammonium sulfate fractionation, P-11, DE-52, Hydroxyapatite, Sephacryl S-200, Blue-Sepharose, DE-52, Phenyl-Sepharose (Shirota and Fujisawa, 1988).

E: Ammonium sulfate fractionation, Butyl-agarose, DEAE-Sephadex, Hexyl-agarose, Sucrose gradient (Srinivasan and Awapara, 1978).

[b]Since this substrate is decarboxylated at a much slower rate than DOPA, valid comparison requires that enzyme units be multiplied by a factor of 5 (Sourkes, 1987).

[c]This substrate is decarboxylated at a higher rate than DOPA, thus the enzyme activity reported is slightly higher (1.4-fold) than when DOPA is used.

[d]The lower incubation temperature for the assay yields lower enzyme activity than that of other procedures.

[e]Specific activity is defined as units/mg protein; one enzyme unit is the amount of enzyme that produces 1 nmole of product (CO_2 or amine) per min at the temperature specified.

TABLE 3 (b)

Purification of DOPA Decarboxylase from Various Tissues

Procedure[a]	F	G	H	I	J	K
Tissue source	Monkey Small Intestine	Human Pheochromocytoma	Rat Liver	Rat Liver	Bovine Adrenals	Bovine Brainstem
Assay conditions						
Substrate	DL-5-HTP[b]	L-DOPA	L-DOPA	L-DOPA	[1-^{14}C]DOPA	L-DOPA
Temperature	37°C	37°C	37°C	25°C[c]	37°C	37°C
pH	8.0	7.2	6.9	6.8	7.0	7.2
Specific activity[d]	35[e]	10,300	5,900	3,330	5,102	43.3
Purification fold	26	3430	790	1189	588	476
Yield (%)	17	33	4.2	19	1.2	9

[a]Procedure F: Protamine sulfate treatment, heat treatment (2 min at 55°C), DEAE-Sephadex (Murali and Radhakrishnan, 1970).

G: Acid treatment (pH 4.7), DEAE-Sephacel, Gel permeation HPLC, Hydrophobic HPLC (phenyl-5PW) (Ichinose *et al.*, 1985).

H: Heat treatment (5 min at 50°C), DEAE-cellulose (twice), Phenyl-Sepharose, Hydroxyapatite, Sephadex G-100 (Ando-Yamamoto *et al.*, 1987).

I: DEAE-BioGel, Phenyl-Sepharose, Hydrophobic HPLC (phenyl-5PW) (Dominici *et al.*, 1987).

J: Ammonium sulfate fractionation, DEAE-cellulose, Sephadex G-200, Hydroxyapatite (Alberts *et al.*, 1987).

K: DEAE-Sephacel, Monoclonal affinity column (Nishigaki *et al.*, 1988).

[b]Since this substrate is decarboxylated at a much slower rate than DOPA, valid comparison requires that enzyme units be multiplied by a factor of 5 (Sourkes, 1987).

[c]The lower incubation temperature for the assay yields lower enzyme activity than that of other procedures.

[d]Specific activity is defined as units/mg protein; one enzyme unit is the amount of enzyme that produces 1 nmole of product (CO_2 or amine) per min at the temperature specified.

[e]This enzyme preparation is only partially purified which accounts for the relatively low enzyme activity.

ammonium sulfate precipitation at 0-4°C is between 25-55%. This will result in a 2- to 4-fold purification over the crude extract. After undergoing various chromatographic steps (most of them are of the open-column type while the more recent procedures have utilized the relatively faster HPLC), the final enzyme preparations were shown to have different specific activities (ranging from 3000 to 11,000 nmoles/min/mg protein) when purified from different tissues. This indicates that either some of the preparations were purified only to apparent homogeneity or partially denatured during purification or that the intrinsic specific activities of the enzyme from different tissues are different. As noted in Tables 3(a) and 3(b), the specific activity of the final enzyme preparations purified by procedures B, E, and F cannot be compared directly with the others since substrates other than DOPA were used. It should also be noted that the enzyme preparation from the small intestine of the monkey was only partially purified and the activity was assayed using DL-5-HTP as substrate (Murali and Radhakrishnan, 1970). This can account for the relatively low specific activity of the enzyme preparation.

4. Properties

Some general properties of DOPA decarboxylase purified from various tissues have been summarized in Table 4. The best characterized DOPA decarboxylase is from the pig kidney. The work has mainly been reported by Borri Voltattorni and co-workers (1987).

(a) *Stability* - DOPA decarboxylase purified from the pig kidney has been reported to be relatively stable in 0.1 M phosphate buffer containing

TABLE 4

Properties of Purified DOPA Decarboxylase

	Pig Kidney[a]	Rat Kidney[b]	Human Pheochromocytoma[c]	Rat Liver[d]	Bovine Adrenals[e]	Bovine Brainstem[f]
Molecular Weight (kDal)	103-112	100-108	100	100	112	100
Subunits (kDal)	50 + 43	2 x 48	2 x 50	2 x 50	2 x 56	2 x 50
Optimum pH	6.8 - 7.0	7.0	7.0	6.8 - 6.9	6.7 - 7.5	7.2
K_m (for DOPA) (M)	1.9×10^{-4}	1×10^{-4}	4.6×10^{-5}	1.2×10^{-4}	1.6×10^{-4}	1.4×10^{-4}
(for 5-HTP) (M)	1.0×10^{-4}	7.0×10^{-5}	6.7×10^{-5}	3.8×10^{-5}	8.3×10^{-5}	1.2×10^{-4}
V_{max}[g] (for DOPA)	8900	9,500	10,300	n.r.[h]	190	43.3
(for 5-HTP)	n.r.	2,100	1550	n.r.	63.3	13.7

[a]From Christenson *et al.* (1970), Lancaster and Sourkes (1972), and Borri Voltattorni *et al.* (1979, 1982).

[b]From Shirota and Fujisawa (1988).

[c]From Ichinose et al. (1986).

[d]From Ando-Yamamoto et al. (1987) and Dominici et al. (1987).

[e]From Albert et al. (1987).

[f]From Nishigaki et al. (1988).

[g]Units in nmoles of product (CO_2 or amine)/min/mg protein.

[h]n.r.= not reported

1 mM dithiothreitol (Borri Voltattorni *et al.*, 1987). There was no loss in enzyme activity for 2 months when stored at -20°C. Nevertheless, this pig kidney decarboxylase showed no loss of activity even when incubated at 25°C for at least 4 hours. In contrast to the findings on the pig kidney enzyme, the partially purified enzyme from the small intestine is extremely labile even in the frozen state (Murali and Radhakrishnan, 1970). Attempts to stabilize the enzyme were not successful and this prevented its further purification.

(b) *Physicochemical properties* - The relative molecular weight (M_r) of the native pig kidney decarboxylase has been reported to range from 85,000 - 90,000 (Lancaster and Sourkes, 1972) to 103,000 daltons (Borri Voltattorni *et al.*, 1979) and 112,000 daltons (Christenson *et al.*, 1970). This kidney enzyme was also shown to be a dimer of two non-identical subunits, with M_r of approximately 43,000 and 50,000 by SDS-polyacrylamide gel electrophoresis (Lancaster and Sourkes, 1972; Borri Voltattorni *et al.*, 1979). However, Christenson *et al.* (1970) suggested the presence of three subunits with the molecular weights of 57,000, 40,000, and 21,000. Moreover, in the rat kidney (Shirota and Fujisawa, 1988), rat liver (Ando-Yamamoto *et al.*, 1987; Dominici *et al.*, 1987), bovine brainstem (Nishigaki *et al.*, 1988), and human pheochromocytoma (Maneckjee and Baylin, 1983; Ichinose *et al.*, 1985), the enzyme (native molecular weight = 100,000) is reported to be composed of two identical subunits with M_r of 50,000. Recently, bovine adrenal DOPA decarboxylase is reported to be a homodimer of a 56,000 dalton monomer subunit (Albert *et al.*, 1987) whereas the rat kidney enzyme is a dimer consisting of identical subunits with M_r of 48,000 dalton (Shirota and Fujisawa,

1988). The reason for the discrepancies is yet to be elucidated. When enzyme preparations from bovine adrenal (Albert *et al.*, 1987), pig kidney (Christenson *et al.*,1970), and rat liver (Dominici *et al.*, 1987) were analyzed by electrophoresis on non-denaturing polyacrylamide gels, most of the enzyme activities were associated with a broad band suggesting a heterogeneous charge distribution. Furthermore, Dominici *et al.* (1987) suggested that the pig kidney and the rat liver DOPA decarboxylases may present different surface hydrophobic sites as their retention times on hydrophobic HPLC were different.

The isoelectric point of the rat liver DOPA decarboxylase was determined to be 5.7 (Ando-Yamamoto *et al.*, 1987) while the purified rat kidney enzyme was resolved into multiple protein bands focusing at the regions of pH 5.6-5.85. Using aromatic L-amino acid decarboxylase partially purified from the rat brain, rat adrenal, and bovine adrenal, isoelectric points of 5.5, 5.5, and 5.0, respectively were obtained (Park *et al.*, 1986). These results also agree with the observation that enzymes from different tissues exhibited heterogeneous charge distribution under non-denaturing conditions as described previously.

The sedimentation constant of the pig kidney enzyme was determined to be 5.82 by Christenson and associates (1970) and 6.4 by Borri Voltattorni and co-workers (1979). The optimum pH for DOPA decarboxylase activity ranges from 6.8 to 7.2 (Table 4). The amino acid composition of the enzyme from various tissues has also been determined (Table 5). DOPA decarboxylase from the pig and rat kidneys, and rat liver have comparable amino acid compositions. In contrast, the enzyme from human pheochromocytoma contains relatively lower amounts of

Table 5

Amino Acid Composition of DOPA Decarboxylase

Amino acid	Residues/molecule			
	Pig Kidney[a]	Rat Kidney[b]	Rat Liver[c]	Human Pheochromocytoma[d]
Aspartic acid[e]	64	54.8	65.4	66.4
Threonine	35	35.1	43.6	40.0
Serine	55	51.1	53.6	160.0
Glutamic acid[e]	107	84.6	100.0	117.3
Proline	50	32.0	44.5	33.6
Glycine	89	55.5	67.3	139.1
Alanine	110	84.8	90.0	85.4
Half-Cystine	21	21.2	26.4	-[f]
Valine	64	54.8	56.4	39.1
Methionine	23	19.4	12.7	25.4
Isoleucine	41	43.7	41.8	29.1
Leucine	125	87.0	96.4	54.5
Tyrosine	28	28.2	21.8	12.7
Phenylalanine	55	50.1	51.8	26.4
Histidine	25	21.5	25.4	22.7
Lysine	48	34.4	47.3	29.1
Arginine	58	57.1	63.6	27.3
Tryptophan	18	12.9	-[f]	-[f]

[a]These values were quoted from Christenson *et al.* (1970).
[b]These values were quoted from Shirota and Fujisawa (1988).
[c]These values were calculated from Dominici *et al.* (1987).
[d]These values were calculated from Ichinose *et al.* (1985).
[e]These figures include both free and amidated residues of aspartic and glutamic acids.
[f]This value was not determined.

arginine, isoleucine, leucine, phenylalanine, and tyrosine and higher amounts of glycine and serine. Since pheochromocytoma is a neoplastic cell, mutations at non-essential residues may account for the difference in amino acid composition. However, this possibility has yet to be explored.

The coenzyme of DOPA decarboxylase is PLP and it is present at 1 mol of PLP per mol of purified enzyme in the pig kidney (Christenson *et al.*, 1970; Borri Voltattorni *et al.*, 1979) and 0.5 mol of PLP per mol of purified enzyme in the rat liver (Dominici *et al.*, 1987).

The absorption spectrum of the asymmetrically enzyme-bound PLP is characterized by 2 maxima at 420 nm and at 330-335 nm (Srinivasan and Awapara, 1978; Borri Voltattorni *et al.*, 1979; Ando-Yamamoto *et al.*, 1987; Dominici *et al.*, 1987). With increasing pH, the ratio of the intensity of these two peaks, A_{335}/A_{420} (3.5-3.8 at pH 6.8) also increases. When PLP was removed by hydroxylamine treatment, the apoenzyme no longer exhibited the absorption maximum at 420 nm but showed a small shoulder at 330 nm (Borri Voltattorni *et al.*, 1971). When fluorescence measurement was made on the holoenzyme, the emission maximum was at 380 nm when excited at 335 nm and a very weak emission occured at 490 nm when the excitation wavelength was 420 nm (Borri Voltattorni *et al.*, 1979).

(c) *Active site* - The amino acid sequence of the phosphopyridoxyl peptide (coenzyme binding site) (Table 6; Morino and Nagashima, 1984), obtained after sodium borohydride ($NaBH_4$) reduction of the holoDOPA decarboxylase and subsequent chymotrypsin digestion, has been determined (Bossa *et al.*, 1977). This sequence shows good homologies with amino

Table 6

Amino Acid Sequence of Phosphopyridoxyl Peptides from Various Amino Acid Decarboxylases

Amino Acid Decarboxylase (Source)	Sequence[a]
Arginine (*E.coli*)[b]	A T H S T H K* L L N A L S Q A S Y
DOPA (Pig Kidney)[c]	N F N P H K* W
Glutamic (*E.coli*)[d]	S I S A S G H K* F
Histidine (*M.morganii* AM-15)[e]	K* M I G S P I P C G I V V A K
Lysine (*E.coli*)[f]	V I Y Q T E S T H K* L L A A F
Ornithine (*E.coli*)[g]	V H K* Q Q A G Q

[a] K*, lysyl residue involved in Schiff base formation with PLP

[b] From Boeker *et al.* (1971).

[c] From Bossa *et al.* (1977).

[d] From Strausbauch and Snell (1970).

[e] From Hayashi *et al.* (1986).

[f] From Sabo and Fischer (1974).

[g] From Applebaum *et al.* (1975).

acid sequences of PLP-binding sites of various bacterial decarboxylases. An interesting finding in this study is the occurrence of a histidine residue immediately adjacent to the α-amino side of the phosphopyridoxyl-lysine residue. It has recently been shown that chemical modification using the histidyl-selective reagent, diethylpyrocarbonate, inhibited pig kidney decarboxylase (Dominici *et al.*, 1985). It was further determined that the modification of one histidyl residue, probably the one adjacent to the phosphopyridoxyl-lysine residue, can inactivate the enzyme. This residue also seems to play an essential role in the catalytic process of decarboxylation. Other amino acid residues at, or near, the active site of the pig kidney DOPA decarboxylase includes an arginine residue (Tancini *et al.*, 1985) and a sulfhydryl residue (Dominici *et al.*, 1984), which are probably involved in the substrate binding site of the enzyme (Borri Voltattorni *et al.*, 1987). The exact position of these residues has yet to be determined as the enzyme has not been sequenced thus far. Only recently, a limited tryptic proteolysis of pig kidney DOPA decarboxylase has been performed and analyzed (Tancini *et al.*, 1988). Tryptic digestion, which inactivated the enzyme, yielded two fragments of M_r 38,000 and 14,000. The PLP-binding site was located in the large fragment of which the NH_2-terminal was blocked. The smaller fragment was determined to be from the COOH-terminus of DOPA decarboxylase. The first 50 amino acid residues of this fragment has been sequenced. The NH_2-terminal of the rat liver enzyme is also blocked (Dominici *et al.*, 1987).

(d) *Substrate specificity* - Aromatic L-amino acid decarboxylase has been suggested to have a broad substrate specificity which included DOPA, 5-HTP, tryptophan, phenylalanine, *p*-tyrosine, and histidine (Lovenberg *et al.*, 1962). In most tissues, the rate of decarboxylation is in that same order (Christenson *et al.*, 1970; Lancaster and Sourkes, 1972; Maneckjee and Baylin, 1983; Ichinose *et al.*, 1985; Shirota and Fujisawa, 1988). In the guinea pig kidney (Srinivasan and Awapara, 1978) and rat liver (Awapara *et al.*, 1962), however, the order of decarboxylation was *o*-tyrosine > DOPA > *m*-tyrosine > 5-HTP. The amino acid *p*-tyrosine was not decarboxylated by the decarboxylase from the guinea pig and pig kidney, and rat liver (Awapara *et al.*, 1962; Lancaster and Sourkes, 1972; Srinivasan and Awapara, 1978). L-*threo*-dihydroxyphenylserine is a substrate for the enzyme purified from the human pheochromocytoma (Ichinose *et al.*, 1985) but not for the pig kidney enzyme (Lancaster and Sourkes, 1972); the latter can also decarboxylate *erythro*-dihydroxyphenylserine. In the study of substrate specificity, using AADC partially purified from the pig kidney and with tryptophan as the substrate, Bosin and colleagues (1974) demonstrated that the "indole nucleus of tryptophan is an absolute requirement for the activity of the enzyme". However, this indole nucleus is not present in substrates like DOPA, dihydroxyphenylserine, tyrosine, and histidine. The kinetic parameters for DOPA and 5-HTP are presented in Table 4. In general, the K_m values for DOPA are 2-5 fold higher than that for 5-HTP and the V_{max} values for DOPA are consistently higher than that for 5-HTP. Aromatic L-amino acid decarboxylase also requires PLP as coenzyme and addition of

10^{-6} - 10^{-4} M of PLP can increase the decarboxylase activity by 2-3 fold.

It should also be noted that the kinetic measurements of DOPA and 5-HTP decarboxylation are complicated by many side reactions including the non-enzymatic decarboxylation of DOPA (Vogel *et al.*, 1970, 1972; Mackowiak *et al.*, 1972) and the non-enzymatic reaction of DOPA and 5-HTP with the free coenzyme, PLP (Schott and Clark, 1952; Tran, 1972; Dominici *et al.*, 1984).

(e) *Inhibitors* - As has been discussed previously (refer to section II.B.1), aromatic L-amino acid decarboxylase is essential for the synthesis of biogenic amines in both the peripheral and central nervous system (CNS). The enzyme is not normally rate-limiting but its inhibition can influence the metabolism of orally or intravenously administered substrates such as L-DOPA and L-5-HTP. DOPA decarboxylase is inhibited by many carbonyl reagents such as cyanide, semicarbazide, hydroxylamine, hydrazine, and some of their derivatives (Sourkes, 1987). These carbonyl reagents have already found clinical use as inhibitors of peripheral DOPA decarboxylase in order to potentiate the central effect of L-DOPA in the treatment of Parkinson's disease. Two of the most important pharmacological agents currently in use, are L-α-methyl-α-hydrazino-3,4-dihydroxyphenylpropionic acid (carbidopa; MK-486) and N-(D,L-seryl-N'-(2,3,4-trihydroxybenzyl)-hydrazine hydrochloride (Ro4-4602; benserazide) (Sourkes, 1977,1979,1987). *In vivo*, these compounds showed marked selectivity for DOPA decarboxylase in the peripheral tissues over that of the CNS (Bartholini *et al.*, 1967). These inhibitors are administered in combination with L-DOPA in the treatment

of Parkinson's disease. Benserazide was discovered by Burkard and co-workers (1962, 1964) who reported that the compound inhibited rat brain and rat kidney decarboxylase activity both *in vivo* and *in vitro*. Carbidopa was discovered in the same year by Porter and colleagues (1962). These investigators have shown that carbidopa inhibited DOPA and 5-HTP decarboxylase activities both *in vivo* and *in vitro*. Using AADC purified from the pig kidney, Borri Voltattorni and Minelli (1977) reported that carbidopa differentially affected enzyme activity using either DOPA or 5-HTP as substrates. Waterhouse and co-investigators (1979) reported that carbidopa at 270 nM inhibited 80% of rat liver DOPA decarboxylase activity. However, since these hydrazine derivatives also interact with other PLP-dependent enzymes, they are not specific for DOPA decarboxylase.

Another pharmacological agent which prompted the synthesis of the above compounds is α-methyl DOPA, a potent antihypertensive agent characterized by Sourkes (1954). This inhibitor is a slow reversible inhibitor of DOPA decarboxylase and is itself a substrate for the enzyme (Sourkes and Rodriguez, 1967). Its decarboxylation in the brain is also shown to be a necessary condition for its hypotensive effect (Rubenson, 1971). Two other analogues of DOPA, α-acetylenic DOPA and α-vinyl DOPA, were also demonstrated to have more pronounced inhibitory effects on DOPA decarboxylase in peripheral tissues than in brain (Ribereau-Gayon *et al.*, 1979). This would make available more DOPA to be transported to the brain for the synthesis of DA, the goal of L-DOPA therapy of Parkinsonian patients. It was hoped that these compounds would complement the administration of L-DOPA, eventually lowering or

maintaining the effective dosage of L-DOPA used and also reducing the incidence of side effects (Bianchine, 1976). However, the effect of α-acetylenic DOPA and α-vinyl DOPA in elevating the brain levels of catecholamines and 5-HT was of too short duration (Ribereau-Gayon *et al.*, 1979). Two additional compounds structurally similar to α-methyl DOPA, *viz.* α-monofluoromethyl DOPA (Jung *et al.*, 1979) and α-difluoromethyl DOPA (Palfreyman *et al.*, 1978; Ribereau-Gayon *et al.*, 1980), were synthesized and found to be potent enzyme-activated irreversible inhibitors of AADC. These so-called 'suicide inhibitors' were found to be no better than carbidopa in increasing the bioavailability of DOPA in the treatment of Parkinson's disease (Huebert *et al.*, 1983). Nonetheless, they were found to be useful tools in studying monoamine turnover (Palfreyman *et al.*, 1984) and as a therapeutic agent for hypertension (Fozard *et al.*, 1980). All of these compounds probably act by virtue of their similarity in structure to the substrate. As suggested by Shikimi and co-workers (1978), the cathecol structure is required for the inhibitory effects on decarboxylation and modification of the side chain changes the potency of inhibition of the compound.

Many other inhibitors of DOPA decarboxylase including NSD 1015, NSD 1034, NSD 1055 (brocresin), MK 785, decaborane, penicillamine, and *p*-toluenesulfonylhydrazine have also been investigated (Kilgallon and Shepherd, 1977; Giorgi and Rubio, 1981). Furthermore, Gey and Giorgi (1974) have shown in a very diverse group of centrally active drugs (thiosemicarbazide, aminooxyacetic acid, pentylenetetrazol, amphetamine) that there is a tendency for drugs which lead to a reduction in DOPA

decarboxylase activity to increase motor activity or induce seizures, whereas anesthetics or depressant drugs are associated with the enhancement of DOPA decarboxylase activity. Treatment with certain organic solvents also enhanced AADC activity towards *p*-tyrosine (Bowsher and Henry, 1983; Juorio and Yu, 1985) and phenylalanine (Juorio and Yu, 1985). However, no increase in AADC activity was observed using DOPA, *m*-tyrosine, or 5-HTP as substrates (Bowsher and Henry, 1983; Juorio and Yu, 1985).

The interaction of vitamin B_6 with a number of drugs has been reviewed (Ebadi *et al.*, 1982; Bhagavan and Brin, 1983). Anti-pyridoxine compounds such as aminooxyacetic acid (AOAA), hydroxylamine, hydralazine, and dihydralazine, have also been used to inhibit DOPA decarboxylase. Aminooxyacetic acid at 10^{-7} to 10^{-4} M did not cause any change in the rat adrenal DOPA decarboxylase activity (Giorgi and Rubio, 1981). At 10^{-3} M, this compound was shown to inhibit 40% of 5-HTP decarboxylase activity while inhibiting 95% of DOPA decarboxylase activity in the rat brain (Sims *et al.*, 1973). Schales and Schales (1949) first reported the inhibition of kidney DOPA decarboxylase activity by hydroxylamine; at 0.05 mM, there was 50% inhibition in 30 min while at 0.1 mM, there was 50% inhibition in 50 min. Meanwhile, the antihypertensive agents, hydralazine (Apresoline) and dihydralazine (Nepresol) have also been shown to inhibit DOPA decarboxylase (Perry *et al.*, 1955, 1969; Schuler and Wyss, 1960). An often prescribed monoamine oxidase inhibitor for the treatment of phobic states, atypical depressions, and posttraumatic stress disorders is phenelzine (Demers *et al.*, 1984). This compound has also been shown to inhibit the

activities of tyrosine aminotransferase and 5-HTP decarboxylase (Dyck and Dewar, 1986).

Recently, Rahman and colleagues (1984) have reported the presence of an endogenous and as yet unidentified inhibitor of AADC in the serum of monkeys. This inhibitor, which was non-dialyzable, completely inhibited enzyme activity with L-5-HTP as substrate while the activity was partially inhibited with L-DOPA as substrate; the inhibition was also reversible.

Finally, DOPA decarboxylase can also be inhibited by some cations. Perry and colleagues (1969) reported that DOPA decarboxylase from guinea pig kidney was inhibited by Cd^{2+} and Hg^{2+} at 0.1 mM concentration, by Cu^{2+} and Zn^{2+} at 1 mM, and by various other cations at 10 mM concentrations. DOPA decarboxylase from the pig kidney was also strongly inhibited by 1 mM concentration of Cu^{2+}, Hg^{2+} and Zn^{2+} (Christenson *et al.*, 1970).

C. How Many Decarboxylases?

Aromatic L-amino acid decarboxylase was first discovered by Holtz and colleagues in 1938 and named DOPA decarboxylase since DOPA was the only amino acid decarboxylated by the enzyme at an appreciable rate (Sourkes, 1979,1987). Later, Clark, Weissbach and Udenfriend (1954) reported the presence of 5-HTP decarboxylase in kidney and liver of many animals. This enzyme which can decarboxylate 5-HTP and DOPA but not tryptophan, phenylalanine, and tyrosine, seemed to be different from

DOPA decarboxylase due to differences in the ratio of the two enzyme activities in various preparations (Table 7) and other properties (Clark *et al.*, 1954). However, studies by Yuwiler and associates (1960) discounted that notion as inhibitors of DOPA decarboxylase also affected 5-HTP decarboxylase. The problem was reinvestigated by Lovenberg, Weissbach, and Udenfriend (1962) who then proposed the name `aromatic L-amino acid decarboxylase' since the enzyme can decarboxylate DOPA, 5-HTP, tryptophan, phenylalanine, *p*-tyrosine, and histidine. This nomenclature was adopted by the IUPAC Commission on Biochemical Nomenclature in 1972 (Sourkes, 1977). However, the question whether there is a single protein species or a group of very closely related proteins such as isozymes is a moot point. This will be addressed in the following discussion.

In studying the distribution of DOPA and 5-HTP decarboxylase activities in cat brain, Kunztman and co-workers (1961) reported that the ratio of the two decarboxylase activities remain essentially constant in various parts of the brain. This was interpreted to mean the same enzyme protein was responsible for the decarboxylations of both DOPA and 5-HTP.

Christenson and his colleagues (1970, 1972) first succeeded in purifying to homogeneity, AADC from the pig kidney and raising a goat antiserum against the purified enzyme. Immunotitrations of this antiserum with extracts from a number of tissues from various species resulted in a proportionate loss of both DOPA and 5-HTP decarboxylase activities (Christenson *et al.*, 1972). Thus, they concluded that DOPA and 5-HTP decarboxylase were indeed a "single enzyme" in all tissues -

Table 7

Relative Activities of DOPA and 5-HTP decarboxylase in Various Preparation of Guinea Pig Kidneys

Preparation	Activity Ratio[a]
Homogenate	6.5
Partially purified 5-HTP decarboxylase	1.4
Acetone powder extract	9.0
Acetone powder extract (Heated 10 min at 55°C)	19.0
Inactivation at pH 5.2	0.7

[a]Values represent the ratio of DOPA decarboxylase activity over 5-HTP decarboxylase activity in guinea pig kidney (Clark *et al.*, 1954).

brain, kidney, liver and pineal - as they were indistinguishable immunologically. However, about ten times more antibody per unit of enzyme activity was required for the complete inhibition of decarboxylase activity in the rat brain compared to that of the pig kidney. Antiserum prepared against purified bovine adrenal DOPA decarboxylase (Goldstein *et al.*, 1973) and rat kidney AADC (Shirota and Fujisawa, 1988) were also demonstrated to inhibit DOPA and 5-HTP decarboxylase activities equally. Additional immunohistochemical studies in rat brain also suggested that the decarboxylases in catecholaminergic and serotonergic neurons were identical (Hokfelt, 1973a,b). However, there remains the possibility that there are distinct but very similar enzymes with similar antigenic regions or that the antibodies are directed toward the common coenzyme, PLP (Sims *et al.*, 1973; Shirota and Fujisawa, 1988). Recently, Ando-Yamamoto *et al.* (1986) demonstrated immunohistochemical and immunochemical cross-reactivity of DOPA decarboxylase and histidine decarboxylase using antibodies produced against these two enzymes. They suggested "the presence of similar antigenic recognition sites inside the native molecules of the two decarboxylases that are exposed when the enzymes are denatured". Furthermore, using immunohistochemical techniques Jaeger *et al.* (1983) has recently shown that certain neurons of the rat CNS contained AADC but not serotonin or tyrosine hydroxylase, the enzyme responsible for the synthesis of DOPA. In addition, variable intensities of AADC immunoreactive products were present in the different group of monoaminergic neurons; serotonergic neurons exhibited the most intense AADC staining, followed by the dopaminergic cells, and

the adrenergic groups (Jaeger *et al.*, 1984). If, as suggested by the authors, the staining intensity of immunoreactive product is indicative of the actual amounts of enzyme present in individual neurons, then the AADC activity in serotonergic neurons would be very high. This would mean that the AADC activity towards DOPA would also be very high since AADC has broad substrate specificity and the rate of DOPA decarboxylation is several times higher than that towards 5-HTP (refer to previous discussion on *Substrate specificity* of AADC: II.B.4.d). If this were not to be the case, then the AADC in serotonergic neurons would exhibit substrate specificity towards only 5-HTP. In view of the contradictory reports, Sourkes (1979) stated that "one could just as well postulate the existence of two homologous enzymes possessing equivalent structure and conformation at the immunocompetent regions of the peptide chain".

Supporting evidence for the "single enzyme" hypothesis has also come from *in vivo* studies by two groups of investigators. Dairman *et al.* (1975) found similar relative decreases of DOPA decarboxylase and 5-HTP decarboxylase activities in the rat brain after intracisternal injections of 6-hydroxydopamine, a drug which selectively destroys catecholaminergic neurons. These investigators also found that the relative loss in the two activities in the spinal cord were similar after intracisternal administration of the serotonergic neurotoxin, 5,6-dihydroxytryptamine. After 6-hydroxydopamine administration, no difference in the two enzyme activities were observed in the spinal cord even though this part of the CNS contains both catecholaminergic and serotonergic neurons (McGeer *et al.*, 1987). In another study (Melamed

et al., 1980) in which unilateral nigrostriatal lesions were produced in the rat by 6-hydroxydopamine, an identical decrease in striatal DOPA decarboxylase and 5-HTP decarboxylase activities was observed. This suggested that the decarboxylase within striatal dopaminergic neurons was nonspecific, using both DOPA and 5-HTP as substrates. In contrast, electrolytic lesions in both median and dorsal raphe nuclei caused a slight but not significant increase in 5-HTP decarboxylase activity - despite large reductions in 5-HT content - but no change in DOPA decarboxylase activity in the striatum (Melamed *et al.*, 1980). This suggested that the decarboxylase in serotonergic neurons in rat striatum was not specific for 5-HTP. Moreover, it has been reported that brain serotonergic neurons can take up DOPA (Barrett and St. Balel, 1971), decarboxylate it to DA and - at least in vitro - release DA in a stimulus-dependent fashion (Ng *et al.*, 1972). These observations can explain the proportionate loss of both DOPA decarboxylase and 5-HTP decarboxylase activities in the rat CNS after administration of neurotoxins, as seen by Dairman *et al.* (1975). The presence of DOPA decarboxylase in serotonergic neurons may serve a modulatory role on 5-HT metabolism and the converse may also be true; however, this has yet to be investigated. The results obtained by Dairman *et al.* (1975) and Melamed *et al.* (1980), conflict with the earlier observations of Sims and Bloom (1973) that intracisternal injection of 6-hydroxydopamine to rats pretreated with pargyline caused a marked decreased in DOPA decarboxylase in upper and lower brainstem regions while not affecting 5-HTP decarboxylase. The activity of 5-HTP decarboxylase increased significantly in hypothalamus, cerebellum, and lateral pons medulla.

Furthermore, following 6-hydroxydopamine treatment, there was a decrease in the ratio of DOPA decarboxylase activity to 5-HTP decarboxylase activity in all regions except the occipital cortex (Sims and Bloom, 1973). If DOPA and 5-HTP decarboxylations are catalyzed by a single protein, the ratio of the two activities should remain relatively constant since both activities were determined using well-defined optimal assay conditions on the same tissue preparation.

By far the best evidence for the "single enzyme" hypothesis has been reported recently by Albert and colleagues (1987). These investigators have purified AADC (DOPA decarboxylase) from bovine adrenal medulla to homogeneity, produced antibodies against it, and isolated the cDNA clone complementary to bovine adrenal AADC mRNA. The authors concluded that a single form of AADC is detected in the rat and bovine tissue as the protein is indistinguishable biochemically and immunochemically in brain, liver, kidney, and adrenal medulla. By *in situ* hybridization with the cDNA, a single 2.3 kilobase mRNA was detected in the bovine adrenal, kidney, and liver while in the rat brain, liver, and pheochromocytoma, the mRNA was 2.2 kilobase in length. Southern blot analysis are also consistent with the presence of a single gene coding for AADC. Interestingly, the authors did not report whether a single mRNA species was present in the bovine brain or the rat adrenal. In addition, the AADC message contains nearly 800 untranslated bases which may yet play a role in defining the specificity of the enzyme in different tissues. As mentioned earlier there may also be immunochemical cross-reactivity between DOPA decarboxylase and other decarboxylases (Ando-Yamamoto *et al.*, 1986).

Evidence for the "dual enzymes" hypothesis in different organs and brain regions has also been reported by a number of investigators. If AADC were a single enzyme, then in pyridoxine deficiency there should be concurrent decreases in both DA and 5-HT levels as AADC is a PLP-dependent enzyme. However, earlier work from this laboratory has found a very significant decrease in brain 5-HT levels of the pyridoxine-deficient growing rat (Dakshinamurti *et al.*, 1976). In contrast, the brain levels of norepinephrine and DA were not altered. Similar observations were obtained in neuronally mature adult rats (Dakshinamurti *et al.*, 1987). Intraperitoneal administration of allylglycine (a convulsant) to mice also did not modify brain DA and norepinephrine contents but did decrease 5-HT content (Sawaya *et al.*, 1978).

The optimal conditions in terms of pH, temperature, substrate and cosubstrate (PLP) concentrations and other properties have been reported to be different for the decarboxylations of DOPA and 5-HTP, respectively (Table 8) (Sims *et al.*, 1973; Sims, 1974). Differences in the kinetic characteristics (K_m and V_{max}) and optimal conditions for DOPA and 5-HTP decarboxylation have also been observed in the guinea pig serum (Rahman *et al.*, 1981a), rat adrenals, liver, and pineal (Rahman *et al.*, 1981b), and human caudate nucleus (Rahman and Nagatsu, 1982). Differential inhibitions of AADC by decarboxylase inhibitors in the brain and liver of cat have also been reported (Bouchard *et al.*, 1981). Bender and Coulson (1972) have also observed in the rat liver that DOPA decarboxylase activity was preferentially inactivated by sodium dodecyl

TABLE 8

Properties and Optimal Experimental Conditions for Rat Brain DOPA and 5-HTP Decarboxylase[a]

	DOPA decarboxylase	5-HTP decarboxylase
1. pH optimum	6.7 - 6.9	8.1 - 8.5
2. Temperature sensitivity	Stable up to 38°C	Increase in activity up to 50°C
3. Pyridoxal phosphate requirement	0.6 mM	0.1 mM
4. K_m for substrate (DOPA or 5-HTP)	0.6 mM	0.016 mM
5. V_{max}	6 μmol/h/g wet wt.	1.02 μmol/h/g wet wt.
6. Presence of ascorbate[b]	No effect	Inhibition
7. Freezing brain in liquid N_2	No effect	No effect
8. Repeated freezing and thawing[c]	Slight Increase	Slight decrease
9. Lyophilization	Increase by 20%	-
10. Subcellular localization	Predominant in the soluble fraction	Distributed almost equally between soluble and particulate fractions

[a]Data are obtained from Sims *et al.* (1973) and Sims (1974).

[b]5.0 mM ascorbate produced 50% inhibition of 5-HTP decarboxylase activity.

[c]Storage in 1 mM PLP is necessary for stability and, after 1 to 2 freeze-thaws, DOPA decarboxylase activity of the preparation is 20% higher than that of the original unfrozen homogenate.

sulfate treatment and 5-HTP decarboxylation by urea; the optimal pH's for the two decarboxylation activities were different as well. However, they concluded that the enzyme has a complex active site, with separate affinities for the two substrates, adjacent to a catalytic site. The selective inhibition of brain AADC by sub-acute α-monofluoromethyl *p*-tyrosine administration has also led to a decrease in brain catecholamines but not of brain 5-HT (Jung *et al.*, 1984). As discussed previously, carbidopa (Borri Voltattorni and Minelli, 1977) and aminooxyacetic acid (Sims *et al.*, 1973) were also shown to differentially inhibit DOPA and 5-HTP decarboxylase activities.

Previous studies have indicated that DOPA decarboxylase is predominant in the soluble fraction of kidney extract (Lovenberg *et al.*, 1962) and the rat brain (Sims *et al.*, 1973). In contrast, there is a substantial proportion (50% or more) of 5-HTP decarboxylase that is associated with membranes (Rodriguez de Lores Arnaiz and DeRobertis, 1964; Sims *et al.*, 1973). Gardner and Richards (1981) also reported that 35% of DOPA decarboxylase was associated with the synaptosomal pool.

The relative distribution of DOPA decarboxylase and 5-HTP decarboxylase has also been investigated in various peripheral tissues and in various regions of the rat brain (Sims *et al.*, 1973; Rahman *et al.*, 1981b). These investigators reported that the activity ratio (DOPA decarboxylase activity/5-HTP decarboxylase activity) varied from one tissue to another and from one brain region to another, which do not agree with the observations by Kunztman *et al.* (1961) who reported a constant ratio of the two decarboxylase activities in different brain

regions of the cat. Furthermore, Saito and colleagues (1975) reported that, in the individual hypothalamic nuclei, the distribution of DOPA decarboxylase correlated directly with those of norepinephrine and dopamine (Palkovits *et al.*, 1974), but inversely with that of serotonin (Saavedra *et al.*, 1974), thus suggesting that DOPA decarboxylase is different from 5-HTP decarboxylase.

DOPA decarboxylase from the rat liver was also described as having different structural properties, surface charge and hydrophobicities, and different antigenic determinants from that of the pig kidney (Dominici *et al.*, 1987). Although the electrophoretic study of AADC from bovine and mouse brain and from human and bovine liver has determined that there is only one molecular species, the data from the rat brain and rat liver showed various degrees of polymerization (Coulson *et al.*, 1969; Cavalli-Sforza *et al.*, 1974). Partially purified aromatic L-amino acid decarboxylase from the rat brain, rat adrenal, and bovine adrenal, have isoelectric points of 5.5, 5.5, and 5.0, respectively (Park *et al.*, 1986). These results tend to agree with the observation that enzymes from different tissues exhibited heterogeneous charge distribution.

Variations in the substrate specificity of AADC has been discussed previously. It was earlier assumed that AADC was also responsible for the synthesis of histamine via the decarboxylation of histidine (Lovenberg *et al.*, 1962) until the identification of a specific histidine decarboxylase in different regions of the brain (Schwartz *et al.*, 1970). Differences in the kinetic characteristics as well as other properties of enzymes catalyzing the same reaction in different

species or even different organs of the same species are known to exist. This has been well illustrated by glutamic acid decarboxylase [EC 4.1.1.15; GAD], sulfinoalanine decarboxylase [EC 4.1.1.29; also known as cysteinesulfinic acid decarboxylase], and histidine decarboxylase [EC 4.1.1.22], all of which are PLP-dependent enzymes involved in the synthesis of γ-aminobutyric acid, taurine, and histamine, respectively. Dissimilarity in the properties of GAD from brain, heart and kidney (Haber *et al.*, 1970; Wu, 1977; Goodyer *et al.*, 1982) have been shown. While the brain GAD is inhibited by anions and carbonyl-trapping agents (e.g. aminooxyacetate) and is stimulated by PLP, the kidney and heart enzymes are stimulated by anions and are unaffected by the addition of PLP. There was also no cross-reactivity immunochemically between the heart, kidney, and liver forms of GAD with the brain GAD (Wu *et al.*, 1978). Further, multiple forms of GAD have been characterized in the pig and rat brain. In the pig brain, there are three forms of GAD, each with the same molecular weight and subunit structure but with distinctly different kinetic constants and pI values (Spink *et al.*, 1985). On the other hand, the rat brain has two forms of GAD that differ in their dependence on PLP, temperature sensitivity, sensitivity to aminooxyacetic acid pretreatment, and electrophoretic mobility (Denner and Wu, 1985). Furthermore, these two forms of rat brain GAD have been demonstrated to be two distinct homodimeric forms of native GAD (Legay *et al.*, 1987). It is also of interest to note that these two forms of rat brain GAD copurified through a variety of chromatographic columns and were not distinguishable immunochemically.

In the late seventies, there was a controversy as to whether GAD was also responsible for the synthesis of taurine by the decarboxylation of cysteic acid. The existence of a specific cysteic acid/cysteine-sulfinic acid decarboxylase (CAD/CSAD) has now been confirmed. This enzyme copurified with GAD through various columns but could be separated by a hydroxyapatite column (Wu, 1982). Both enzymes are also immunologically distinct. Cysteic acid, cysteinesulfinic acid, and glutamic acid are all substrates for GAD but only the former two are substrates for CAD/CSAD with a different K_m for each substrate. Multiple forms of CSAD were also found in the rat liver (Weinstein and Griffith, 1987).

Histidine decarboxylase from the rat brain and peripheral tissues (rat stomach, rat whole fetus, rat liver, and rat gastric mucosa) have certain properties such as optimal pH, molecular weight and antigenic properties in common, merely to differ in their substrate and coenzyme binding affinities and isoelectric points (Palacios *et al.*, 1976; Savany and Cronenberger, 1982a,b; Taguchi *et al.*, 1984; Yamada *et al.*, 1984). Histidine decarboxylase purified from mouse kidney also do not share the properties of the enzyme from other tissues (Martin and Bishop, 1986).

It is evident from the above discussion that it is not always possible to infer the characteristics of neuronal enzymes by extrapolating results from the non-neuronal enzymes. The suggested identity of DOPA and 5-HTP decarboxylases is based on evidence extrapolated from studies on non-neuronal enzymes. One of the more compelling reasons for investigating DOPA and 5-HTP decarboxylases is the detection of immunochemical cross-reactivity between DOPA

decarboxylase and HDC (Ando-Yamamoto *et al.*, 1986). The same may be true for DOPA and 5-HTP decarboxylases. Likewise, the overlapping substrate specificities of GAD and CAD/CSAD may also apply to DOPA decarboxylase and 5-HTP decarboxylase, each with different affinities for different substrates. The use of 5-HTP or DOPA interchangeably as substrates for the determination of DOPA decarboxylase or 5-HTP decarboxylase activities may well provide misleading information. There is at present "no complete agreement on the singular character of aromatic amino acid decarboxylases in mammalian tissues" (Sourkes, 1987). This question must be resolved by complete purification and sequence analysis of the respective decarboxylase(s) involved, from neuronal and non-neuronal tissues. The possibility for multiple molecular forms of AADC with different substrate specificity should also be considered.

D. Pyridoxine deficiency

1. Pyridoxine and the Nervous System

Available information on the role of pyridoxine in the development and function of the nervous system has been reviewed (Dakshinamurti, 1982; Dakshinamurti *et al.*, 1985a, 1988). Many of the effects of pyridoxine deficiency can be explained on the basis of specific decreases in certain decarboxylases (glutamic acid, cysteinesulfinic acid, ornithine, and 5-hydroxytryptophan) (Figure 6). Other effects

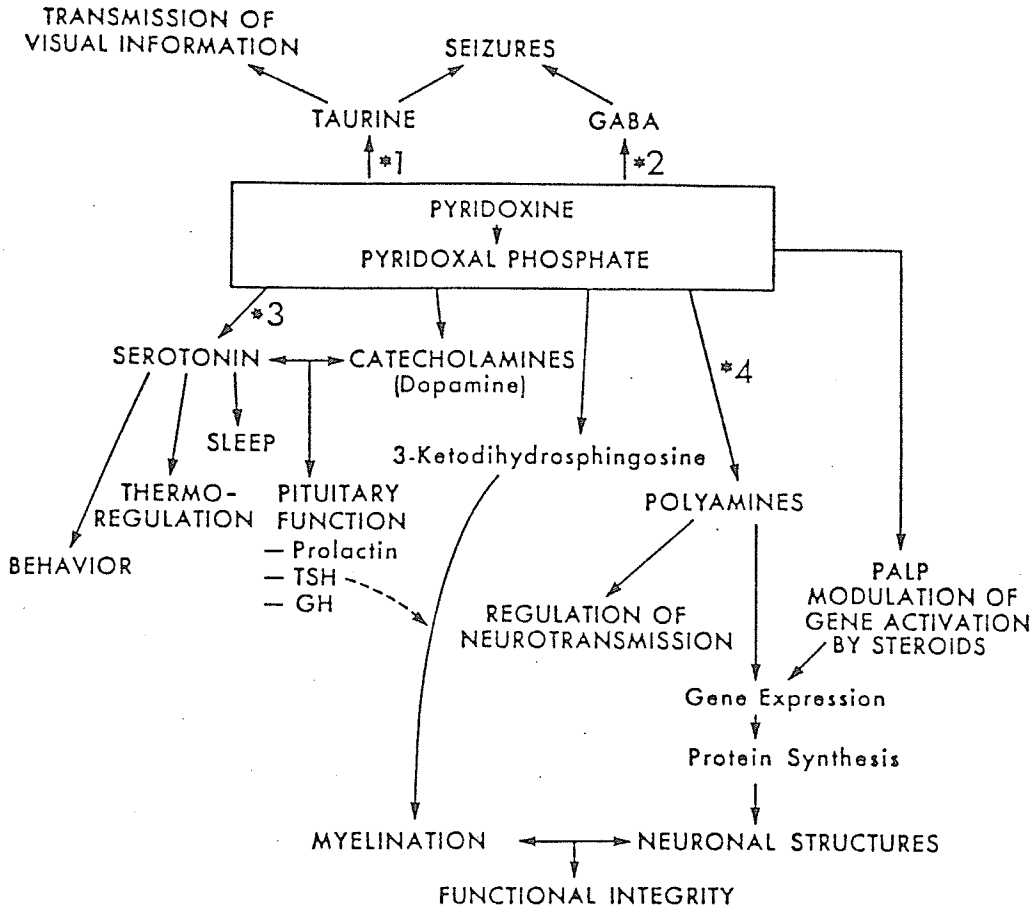

Figure 6. Involvement of pyridoxine in CNS [*1=Cysteinesulfinic acid decarboxylase, *2=glutamic acid decarboxylase, *3=5-hydroxy-tryptophan decarboxylase, *4=ornithine decarboxy-lase] (Dakshinamurti, 1982).

like the impairment of myelination seem to be more complex involving endocrine abnormalities induced by pyridoxine deficiency.

2. Experimental Pyridoxine Deficiency

Dakshinamurti and Stephens (1969) first reported the production of congenital pyridoxine deficiency. It was later showed that a deficiency of pyridoxine in rat pups could be produced by depriving the dam of dietary pyridoxine during lactation (Stephens *et al.*, 1971). This decreased the mortality of the pyridoxine-deficient rat pups and facilitated the study of the role of pyridoxine during development. These studies have characterized pyridoxine deficiency in biochemical and electrophysiological terms in young rats which have been subjected to this stress during the critical period of CNS development. Rats fed the pyridoxine deficient diet exhibited significantly lower levels of PLP and γ-aminobutyric acid (GABA) in various brain regions. The activities of whole brain GAD and CAD/CSAD were decreased whereas the activity of GABA-transaminase (GABA-T) was not affected by pyridoxine-deficiency (Dakshinamurti *et al.*, 1985). This was ascribed to a greater affinity of GABA-T for PLP. In a study of the oxidative reactions, there was no difference between mitochondria prepared from brains of pyridoxine-deficient and pyridoxine-supplemented neonates in terms of oxygen consumption, ADP/oxygen, as well as the concentration of the respiratory carriers (Bhuvaneswaran and Dakshinamurti, 1972). The electroencephalogram (EEG) of pyridoxine-deficient animals showed spike activity, presumably indicative of seizures in deficient animals, caused by the decrease in GABA. Evoked potentials presented abnormalities in

their latency, wave form and response to repetitive stimuli and the extent to which they were affected depended on the intensity of the deficiency. The changes observed in the deficient animals were the result of retardation of normal ontogenetic development of the CNS of these animals (Stephens *et al.*, 1971).

The effects of pyridoxine deficiency in the neuronally mature adult rat were also examined (Dakshinamurti *et al.*, 1987). The results indicated that the induction of pyridoxine-deficiency in the adult rat does not produce the electrophysiological effects and defective myelination that are seen when pyridoxine-deficiency was induced during the CNS maturation period. These deficient animals also show decreased levels of GAD activity in various brain regions, which resulted in decreased GABA levels (Paulose and Dakshinamurti, 1984; Paulose *et al.*, 1988).

3. Monoamine levels

The possible sites of regulation of the synthesis of various monoamines are: availability of the precursor amino acid at the neuronal site (Wurtman *et al.*, 1980; Fernstrom, 1983), hydroxylation (Levitt *et al.*, 1965), and decarboxylation steps (Figure 7).

Aromatic L-amino acid decarboxylase has long been implied to be a single enzyme being responsible for the decarboxylations of the precursors of monoamines in both neuronal and non-neuronal tissues. Since this enzyme is PLP-dependent, one would expect a concurrent decrease in the levels of both 5-HT and catecholamines in the brain of pyridoxine-deficient rats. Regardless of the method used to deplete

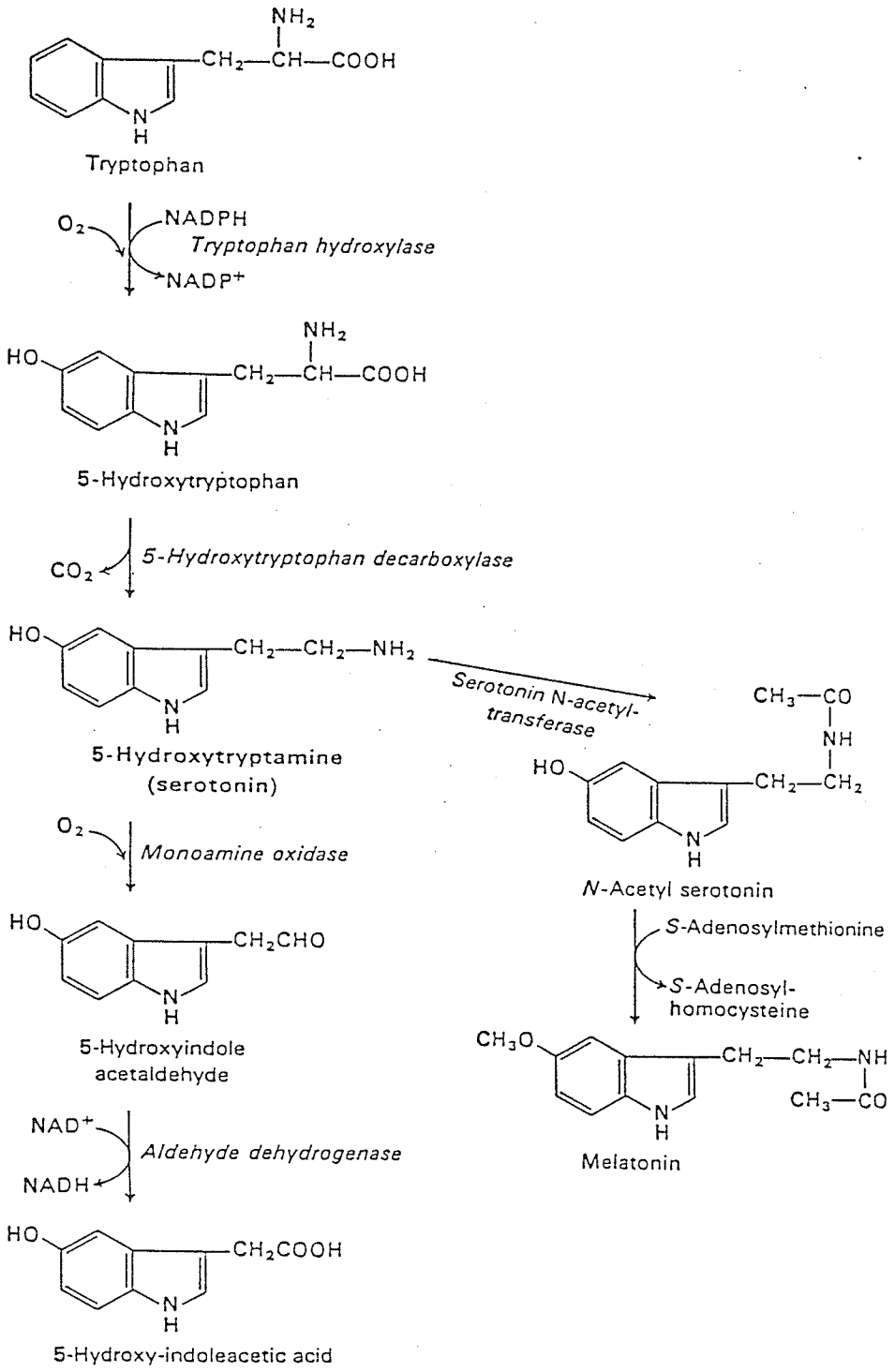

Figure 7. The biochemical pathway of serotonin metabolism.

pyridoxine - dietary or using antimetabolites like 4-deoxypyridoxine or penicillamine - a very significant decrease in the brain 5-HT content was seen in pyridoxine-deficient young rats with no change in brain catecholamines (Dakshinamurti *et al.*, 1976; LeBlancq, 1979). Similar results were obtained in various brain regions of the pyridoxine-deficient young rats (Dakshinamurti *et al.*, 1985b; Paulose and Dakshinamurti, 1985) and adult rats (Dakshinamurti *et al.*, 1987). Sourkes (1972) also reported that there was no change in the steady state concentration of epinephrine and norepinephrine in various tissues in the pyridoxine-deficient rats. There was also no difference in the excretion in urine of endogenous metabolites of DA between the pyridoxine-deficient and pyridoxine-supplemented rats. The possibility that the decrease in brain 5-HT seen in pyridoxine-deficient rats was a result of inanition and the generalized malnutrition was methodically ruled out. Further metabolism of 5-HT by monoamine oxidase or the transport of its metabolite, 5-hydroxyindoleacetic acid to cerebrospinal fluid were also not altered in pyridoxine deficiency. In addition, the levels of brain tryptophan or the activity of tryptophan hydroxylase were unaffected by pyridoxine deficiency. The site of difference between the pyridoxine-deficient and control rats is the decarboxylation step (Dakshinamurti *et al.*, 1976; Dakshinamurti, 1982).

4. Consequences of Decreased Brain Serotonin

The physiological consequences of the decrease in 5-HT in the pyridoxine-deficient rat brain have been reviewed (Dakshinamurti, 1982; Dakshinamurti *et al.*, 1985a, 1988).

The consistent decrease in deep body temperature seen in the pyridoxine-deficient rats (Dakshinamurti *et al.*, 1976) may be related to the deficit in brain 5-HT content. Myers (1975) has presented evidence to support a serotonergic mechanism in the hypothalamus as being involved in thermoregulation in the rat. Pyridoxine deficiency also affects sleep pattern in rats (Dakshinamurti, 1982). The duration of deep slow-wave sleep (SWS 2) and REM is shortened and in some instances completely abolished. These animals are in shallow slow-wave sleep (SWS 1). These effects on sleep parallel that of experimental serotonin deficiency in animals and man, which corroborate the hypothesis by Jouvet (1972) that serotonergic neurons play a major role in the maintenance of slow-wave sleep 2 and REM events.

The central regulation of thyroid hormone secretion by monoamine neurotransmitters through the hypothalamic-pituitary pathway has been reported by many different investigators. In view of this, Dakshinamurti and associates (1985b, 1986) have studied the thyroid function in pyridoxine-deficient rats. In pyridoxine-deficient rats, hypothalamic 5-HT was significantly decreased whereas the catecholamine levels were unaltered (Dakshinamurti *et al.*, 1985b). Although significantly reduced levels of serum triiodothyronine and thyroxine and pituitary thyroid stimulating hormone (TSH) were found in the deficient rat, the pituitary response to thyroid releasing hormone (TRH) was not impaired. The results from these studies can be interpreted as consistent with a hypothalamic type of hypothyroidism in the pyridoxine-deficient rat caused by the specific decrease in 5-HT.

The roles of 5-HT and GABA in the central regulation of blood pressure have been studied (DeJong *et al.*, 1975; Persson, 1980). Hypothyroidism is also known to cause hypertension (Saito *et al.*, 1983). Since both hypothyroidism and decreased 5-HT and GABA levels have been observed in the pyridoxine-deficient rats, the sympathetic stimulation and hypertension occurring in pyridoxine deficiency was investigated (Paulose *et al.*, 1988). In pyridoxine-deficient adult rats, hypertension was associated with sympathetic stimulation. Treatment of pyridoxine-deficient rats with a single dose of pyridoxine was shown to reverse the blood pressure to normal levels within 24 hours, with concomitant restorations of hypothalamic 5-HT, GABA, as well as the return of plasma epinephrine and norepinephrine to normal levels. The decreased serotonergic and GABAergic central neurotransmission in the pyridoxine-deficient rat, acting through stimulation of sympathetic outflow, has been suggested to cause the reversible hypertension seen in this animal model.

The highest concentration of 5-HT in the body is in the pineal gland (Quay and Halevy, 1962) where 5-HT is ultimately converted to melatonin, via the methylation of N-acetylserotonin (NAS). The rate-limiting step in melatonin synthesis is believed to be the production of NAS (Klein and Weller, 1973) but it is not always so. Decreases in pineal 5-HT, 5-hydroxyindoleacetic acid, NAS, and melatonin were seen in moderately pyridoxine-deficient adult rats (Viswanathan *et al.*, 1988). It was further determined that 5-HT availability could be an important factor in the regulation of NAS and melatonin synthesis in the pineal.

Hence, the impairment of pineal function caused by pyridoxine deficiency may have pathophysiological consequences.

The above discussion delineates the importance of investigating the enzyme or enzymes responsible for the decarboxylation of DOPA and 5-HTP in the brain. A better comprehension of the nature and properties of the enzyme responsible for DOPA decarboxylation has pharmacological significance as it may lead to better intervention procedures in the treatment of Parkinson's disease.

CHAPTER III

EXPERIMENTAL SECTION I (PART A)

EFFECT OF PYRIDOXINE DEFICIENCY ON AROMATIC L-AMINO ACID DECARBOXYLASE IN ADULT RAT BRAIN

Effect of pyridoxine deficiency on aromatic L-amino acid decarboxylase in adult rat brain

A. Introduction

Pyridoxal phosphate (PLP) is the major coenzymic form of pyridoxine. The participation of PLP in the catabolism of many amino acids has drawn major research interest throughout the years. The decrease in activity of many of the PLP-dependent decarboxylases has been the landmark on which many explanations of the clinical effects of pyridoxine deficiency are based (Dakshinamurti, 1982). For example, the involvement of glutamic acid decarboxylase and γ-aminobutyric acid in the etiology of certain convulsive seizures have been recognized.

Aromatic L-amino acid decarboxylase is also a PLP-dependent enzyme. In view of its lack of substrate specificity, it has been generally accepted that it is involved in the formation of both the catecholamines and serotonin (5-HT). Hence, in pyridoxine deficiency parallel decreases in brain catecholamines and 5-HT levels would be expected. However, a significant decrease in brain 5-HT with no alteration in the level of brain dopamine (DA) and norepinephrine in the pyridoxine-deficient young rats (Dakshinamurti *et al.*, 1976) and adult rats (Dakshinamurti *et al.*, 1987) has been reported. It was shown that this decrease in 5-HT in the pyridoxine-deficient rat brain could not be ascribed to a decrease either in the brain tryptophan content or in the activity of tryptophan hydroxylase. Further metabolism of 5-HT and the transport of its metabolite, 5-hydroxyindoleacetic acid to cerebrospinal

fluid were not altered in pyridoxine deficiency. We have now investigated the decarboxylation step using both DOPA and 5-HTP as substrates in normal and pyridoxine-deficient adult rat brain.

B. Experimental

1. Materials

L-3,4-Dihydroxyphenyl[3-^{14}C]alanine (sp. act. 10.9 mCi/mmol) and 5-hydroxy[side chain-2-^{14}C]tryptamine creatinine sulfate (sp. act. 59 mCi/mmol) were purchased from Amersham Corporation (Oakville, ON). DL-5-[3-^{14}C]-hydroxytryptophan (sp. act. 59.0 mCi/mmol) and [1-^{14}C]-3,4-dihydroxyphenylethylamine hydrobromide (sp. act. 15.5 mCi/mmol) were obtained from New England Nuclear Corp. (Mississauga, ON). The scintillant cocktails, Aquasol-2 and Scintiverse II, were from New England Nuclear Corp. and Fisher Scientific Co. (Edmonton, AB) respectively. Pargyline (N-benzyl-N-methylpropargylamine) hydrochloride was from Saber Laboratories, Inc. (Morton Grove, IL). All other chemicals and reagents were obtained either from Sigma Chemical Co. (St. Louis, MO) or from Fisher Scientific Co. (Edmonton, AB).

2. Production of pyridoxine-deficiency

Weaned male Sprague-Dawley rats (45-50g) were purchased from Charles River Canada (St. Constant, PQ). They were divided into two groups, one was fed a pyridoxine-deficient diet (Dakshinamurti and Stephens, 1969) while the other was fed the same diet but supplemented with pyridoxine (100 mg/kg diet). The composition of the diet is given in Tables 9 and 10. Animals were housed individually and were provided with diet and water *ad libitum*. They were killed by decapitation when they were 16 weeks old, at which time morphological symptoms of pyridoxine deficiency were apparent. These include acrodynia which is

TABLE 9

Percentage Composition of Pyridoxine Deficient Diet

Vitamin free casein	30.00 %
Dextrose	59.85
Corn oil	5.00
Rogers & Harper salt mixture (ICN)	4.00
Vitamin mixture (without pyridoxine)[a]	1.00
Choline chloride	0.15
	100.00 %

[a]See Table 10 for composition of pyridoxine-deficient vitamin mixture.

TABLE 10

Composition of Pyridoxine Deficient Vitamin Mixture

p-Aminobenzoic acid	30.00 g
Ascorbic acid	99.20
Biotin	0.05
Calcium pantothenate	6.60
Folic acid	0.20
Inositol	11.00
Menadione	5.00
Niacin	9.90
Riboflavin	2.20
Thiamine HCl	10.00
alpha-Tocopherol (250 I.U./g)	10.00
Vitamin A (250,000 Units/g)	8.00
Vitamin B_{12} (Triturate in 0.1% Mannitol)	3.00
Vitamin D (500,000 Units/g)	0.04
Dextrose	823.81
	1000.00 g

characterized by hyperkeratosis and loss of subcutaneous fat, lesions on the snout, tail and eye areas, and also hair loss around the snout (Singer, 1981; Dakshinamurti *et al.*, 1987). The brain was quickly removed and the regions dissected according to Glowinski and Iversen (1966). Tissues were frozen immediately on dry ice, stored at -70°C, and analyzed for decarboxylase activities within two months. Activity of tissues frozen for varying periods of time up to three months, were comparable to the activity of fresh tissue.

3. Tissue homogenization

For the determination of DOPA decarboxylase activity, tissues were homogenized in ice-cold 80 mM sodium phosphate, pH 6.7, containing 10 mM 2-mercaptoethanol, with a Potter-Elvehjem type homogenizer. A similar homogenate was prepared with 75 mM Tris-HCl, pH 8.3, containing 10 mM 2-mercaptoethanol for the determination of 5-HTP decarboxylase activity. All homogenates were used immediately.

4. Preparation of pyridoxal 5'-phosphate depleted brain homogenate

Adult male Sprague-Dawley rats (150-200 g) were killed by decapitation and their brain quickly removed, frozen on dry ice, and stored at -70°C. Whole brain homogenates were prepared as described in the previous section. Endogenous PLP was removed by hydroxylamine treatment (Lipson *et al.*, 1980). An aliquot of the homogenate for DOPA decarboxylase assay was dialyzed at 4°C against sodium phosphate buffer, pH 6.7, containing 10 mM 2-mercaptoethanol and 5 mM hydroxylamine for 60 min, followed by dialysis twice against the same buffer but without

hydroxylamine for 30 min each. A different aliquot of the untreated homogenate was dialyzed twice against the same buffer but without hydroxylamine at 4°C for 60 min each. The treated and untreated homogenates were then assayed for DOPA decarboxylase activity with and without the *in vitro* addition of PLP. Homogenates for 5-HTP decarboxylase assay were dialyzed similarly using Tris-HCl buffer, pH 8.3 and assayed with and without the *in vitro* addition of PLP.

5. DOPA decarboxylase assay

DOPA decarboxylase was assayed using the methods of Christenson *et al.* (1970) and Laduron and Belpaire (1968) with modifications. The preincubation medium contained, in a total volume of 0.8 ml: 80 μmoles of sodium phosphate, pH 6.7; 0.125 μmoles of PLP; and enzyme. The amount of protein used was 0.8-1.1 mg for brainstem, cerebellum, corpus striatum and hypothalamus, and 1.5-2.0 mg for cerebral cortex and whole brain. This was preincubated at 37°C for 5 min in a metabolic shaker. Then 0.2 ml of 5 mM L-DOPA, containing 0.1 μCi of L-[3-^{14}C]-DOPA, was added to initiate the enzyme reaction. The blank was the same except that buffer or heat-denatured enzyme was used in place of the enzyme. The samples were incubated at 37°C for 15-20 min. Thereafter, the mixtures were immediately chilled on ice and excess sodium chloride (1 g) to saturate the medium was added. Then the mixture was extracted three times, each with 2 ml of 1-butanol. Radioactive DOPA was minimally extracted into butanol at pH 6.7 due to presence of a charged α-carboxyl group, which makes it more soluble in the aqueous fraction than in non-aqueous (butanol) fraction. From the pooled butanol

extracts, a 1.2 ml aliquot was mixed with Aquasol-2 or Scintiverse II and counted in a Beckman LS 3801 Liquid Scintillation System. All samples were assayed in duplicate. By carrying out the extraction procedure with [1-^{14}C]-3,4-dihydroxyphenylethylamine (dopamine), the recovery was found to be 73.7 $\pm$ 7.6% (mean $\pm$ S.D.; n=11). DOPA decarboxylase activity was expressed as pmoles of dopamine formed/min/mg protein. The limit of detection was 83 pmoles/min/mg protein. Holoenzyme activity was determined by using buffer in place of PLP in the preincubation medium. Since no significant difference in enzyme activity was observed in the presence or absence of pargyline during the 15 min incubation period, no monoamine oxidase inhibitor was added to the incubation medium for this assay in further studies.

6. 5-HTP decarboxylase assay

5-HTP decarboxylase was assayed using the methods of Sims *et al.* (1973) and Kuntzman *et al.* (1961) with modifications. The preincubation medium contained, in a total volume of 0.8 ml: 75 µmoles of Tris-HCl, pH 8.3; 0.3 µmoles of PLP; 0.1 µmole of pargyline hydrochloride; and enzyme. The amount of protein used was 0.6-0.8 mg for brainstem, cerebellum, corpus striatum and hypothalamus, and 1.3-1.7 mg for cerebral cortex and whole brain. This was preincubated at 37^{o}C for 5 min in a metabolic shaker. Then 0.2 ml of 3 mM DL-5-HTP containing 0.1 µCi of DL-[3-^{14}C]-5-HTP, was added to initiate the enzyme reaction. The blank was the same except that buffer or heat-denatured enzyme was used in place of the enzyme. The samples were incubated at 37^{o}C for 60 min. Thereafter, the mixtures were immediately chilled on ice and

excess sodium chloride (1 g) to saturate the medium was added, followed by 1 ml of saturated sodium carbonate, pH 11. This was extracted twice, each with 3 ml of 1-butanol. The butanol extract in each case was washed with 2 ml of sodium chloride-butanol saturated borate buffer (0.5 M, pH 10) to remove 5-HTP. From the pooled butanol extracts, a 1.2 ml aliquot was mixed with Aquasol-2 or Scintiverse II and counted in a Beckman LS 3801 Liquid Scintillation System. All samples were assayed in duplicate. By carrying out the extraction procedure with 5-hydroxy-[side chain-2-^{14}C]-tryptamine (5-HT), the recovery was found to be 83.8 $\pm$ 5.4% (mean $\pm$ S.D.; n=10). 5-HTP decarboxylase activity was expressed as pmoles of serotonin formed/min/mg protein. The limit of detection was 0.6 pmoles/min/mg protein. Holoenzyme activity was determined by using buffer in place of PLP in the preincubation medium.

7. Measurement of protein

Protein contents were determined using Bio-Rad Protein Assay which is based on the method of Bradford (1976) with bovine serum albumin as a reference standard.

C. Results

Whole brain rates of activity for DOPA decarboxylase and 5-HTP decarboxylase, respectively, were 774 ± 71 and 56.5 ± 5.9 pmol/min/mg protein (mean ± S.E.M.; n=10).

1. Characterization of DOPA decarboxylase activity

Under our assay conditions, the optimal pH for the determination of DOPA decarboxylase activity was found to be pH 6.7 (Figure 8). Maximal rate of decarboxylation of DOPA was obtained with the addition of 0.125 mM PLP (Figure 9). At concentrations higher than this, a reduction in DOPA decarboxylase activity was observed. This was probably due to the depletion of the substrate, DOPA which has been reported to react non-enzymatically with PLP to form a corresponding tetrahydroisoquinoline (Schott and Clark, 1952). When increasing levels of PLP was incubated with DOPA along with the rest of the reaction mixture with the exception of the enzyme, a linear decrease in the amount of DOPA extracted into the butanol phase was observed. In the extraction with butanol, it was found that when no PLP was present in the reaction mixture 13.1% of DOPA was extracted and this was decreased to 5.8% in the presence of 1 mM PLP. Thus, the expected tetrahydroisoquinoline condensation product (Schott and Clark, 1952) seems to be more soluble in the aqueous phase of the reaction mixture.

Maximal rate of decarboxylation was observed with 1 mM DOPA. At DOPA concentration greater than 2.5 mM, substrate inhibition was observed. DOPA decarboxylase activity was proportional to enzyme

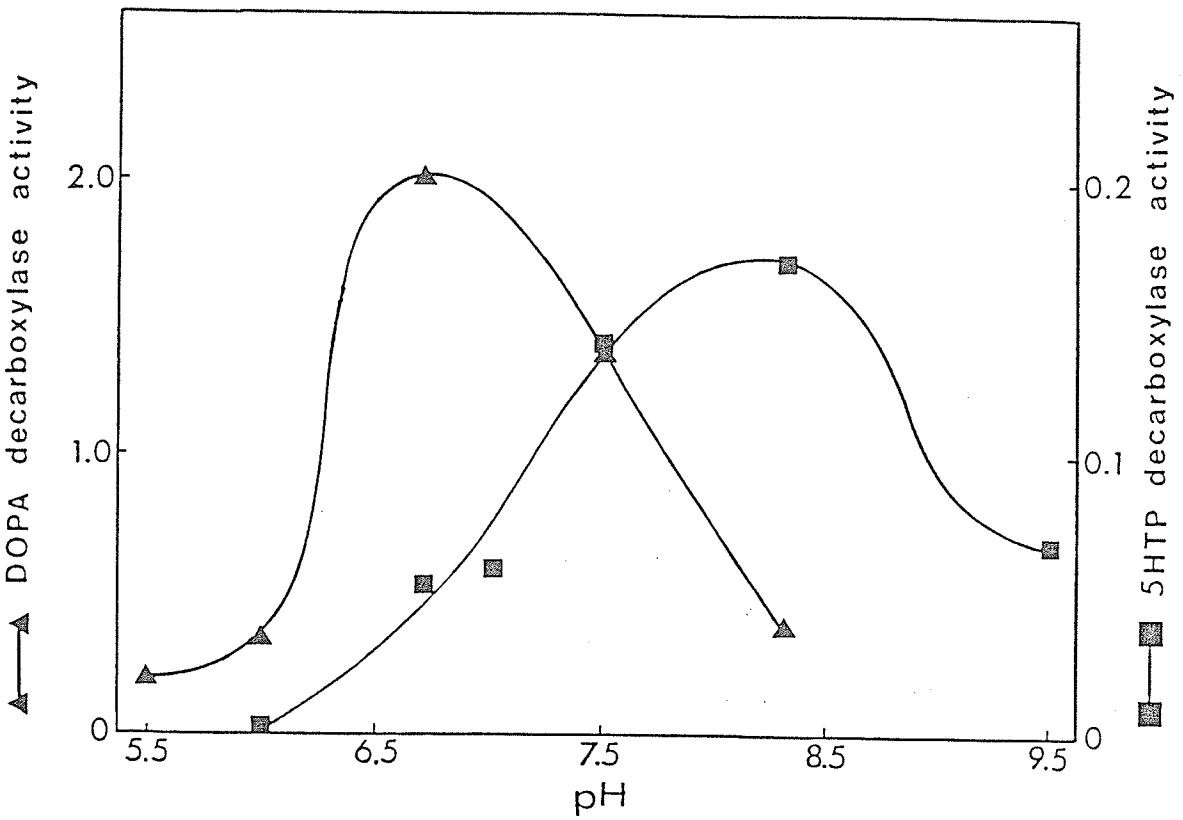

Figure 8. The activities of brain DOPA decarboxylase and 5-HTP decarboxylase at varying pH. Sodium phosphate buffer was used for assays at pH 5.5-7.5 while Tris-HCl was used for assays at pH 7.5-9.5. Enzyme activities are expressed as nmol/min/mg protein.

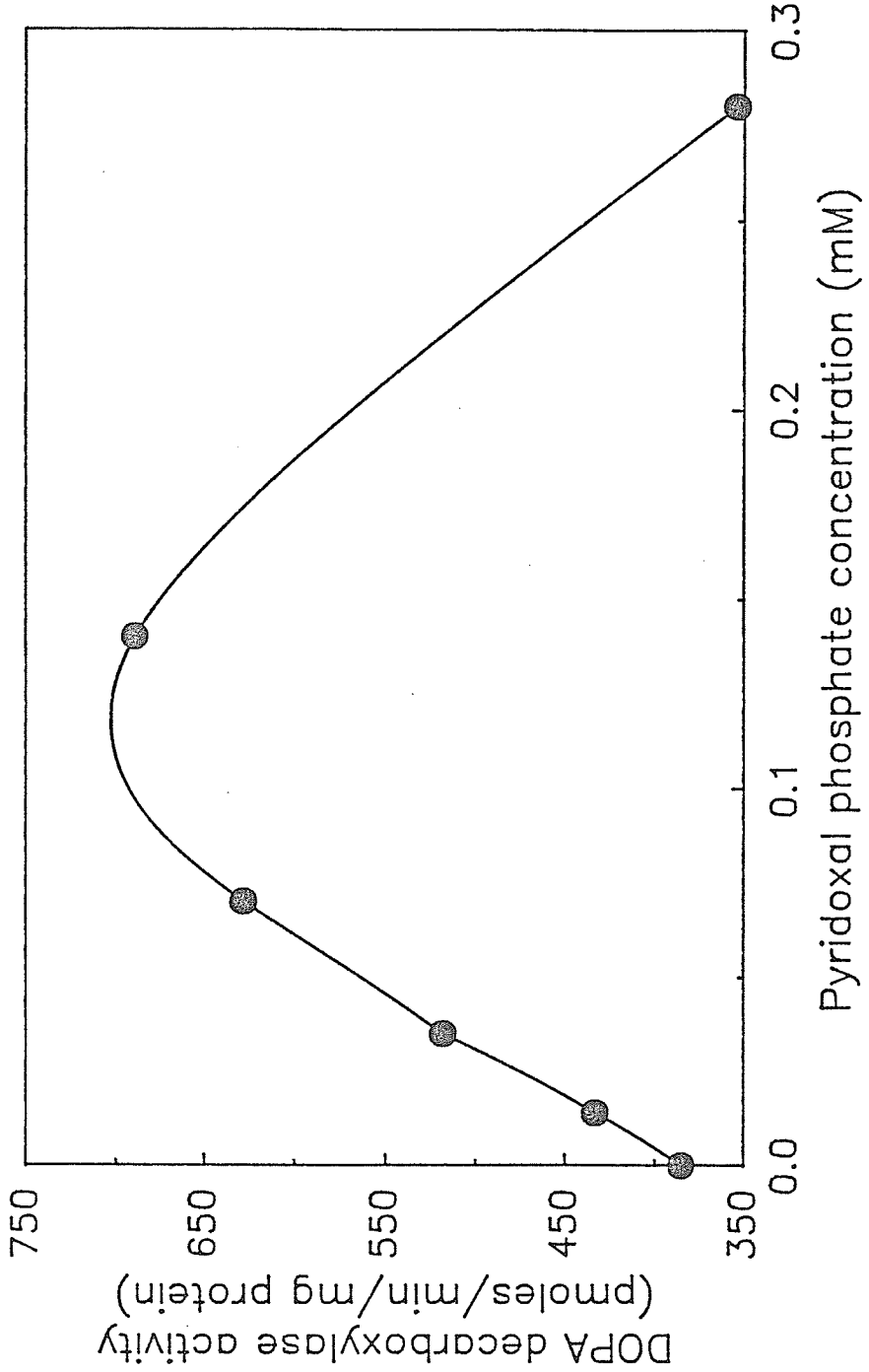

Figure 9. Effect of PLP on rat brain DOPA decarboxylase activity. DOPA decarboxylase activity was measured as described in Chapter III Section I (Part A).B.5, except that the concentration of PLP was varied as indicated.

protein concentration up to 4.56 mg/ml and was linear up to 60 min incubation time. The K_m for both DOPA and PLP are given in Table 11.

2. Characterization of 5-HTP decarboxylase activity

Optimal rate of decarboxylation of 5-HTP to 5-HT was at pH 8.3 (Figure 8) and at a PLP concentration of 0.3 mM (Figure 10). At higher PLP concentrations, a reduction in 5-HTP decarboxylase activity was observed. The rate of decarboxylation was found to be optimal with 0.6 mM 5-HTP. Substrate inhibition was observed at higher 5-HTP concentration. 5-HTP decarboxylase activity was proportional to enzyme concentration up to 3.75 mg/ml and was linear up to 120 min incubation time. The K_m for both 5-HTP and PLP are given in Table 11.

3. Regional distribution of DOPA decarboxylase and 5-HTP decarboxylase

The gross regional distribution of DOPA and 5-HTP decarboxylase in 5 regions of the normal rat brain is given in Table 12. In all regions, DOPA decarboxylase activity was higher than 5-HTP decarboxylase activity. When the ratios of activities were compared, as much as 4-fold difference in the activity of DOPA decarboxylase relative to that of 5-HTP decarboxylase was observed.

4. Effect of pyridoxine deficiency

Under optimal conditions of substrate and PLP concentration, a significant decrease in DOPA decarboxylase activity was observed in the brainstem ($p<0.001$), cerebellum ($p<0.025$), cerebral cortex (0.001), and corpus striatum ($p<0.001$) of the pyridoxine-deficient rats when compared

TABLE 11

Kinetic Parameters for Rat Brain DOPA and 5-HTP Decarboxylase

	DOPA decarboxylase	5-HTP decarboxylase
1. K_m for substrate (DOPA or 5-HTP) (M)	1.65×10^{-4}	2.70×10^{-4}
2. V_{max} (pmoles/min/mg protein)	1437	99.7
3. K_m for PLP (M)	9.44×10^{-6} *a*	2.34×10^{-4} *a*
	5.47×10^{-5} *b*	2.30×10^{-3} *b*

[a]Data determined from undialyzed whole brain homogenate.

[b]Data determined from PLP-depleted brain homogenate as described in Chapter III Section I (Part A).B.4

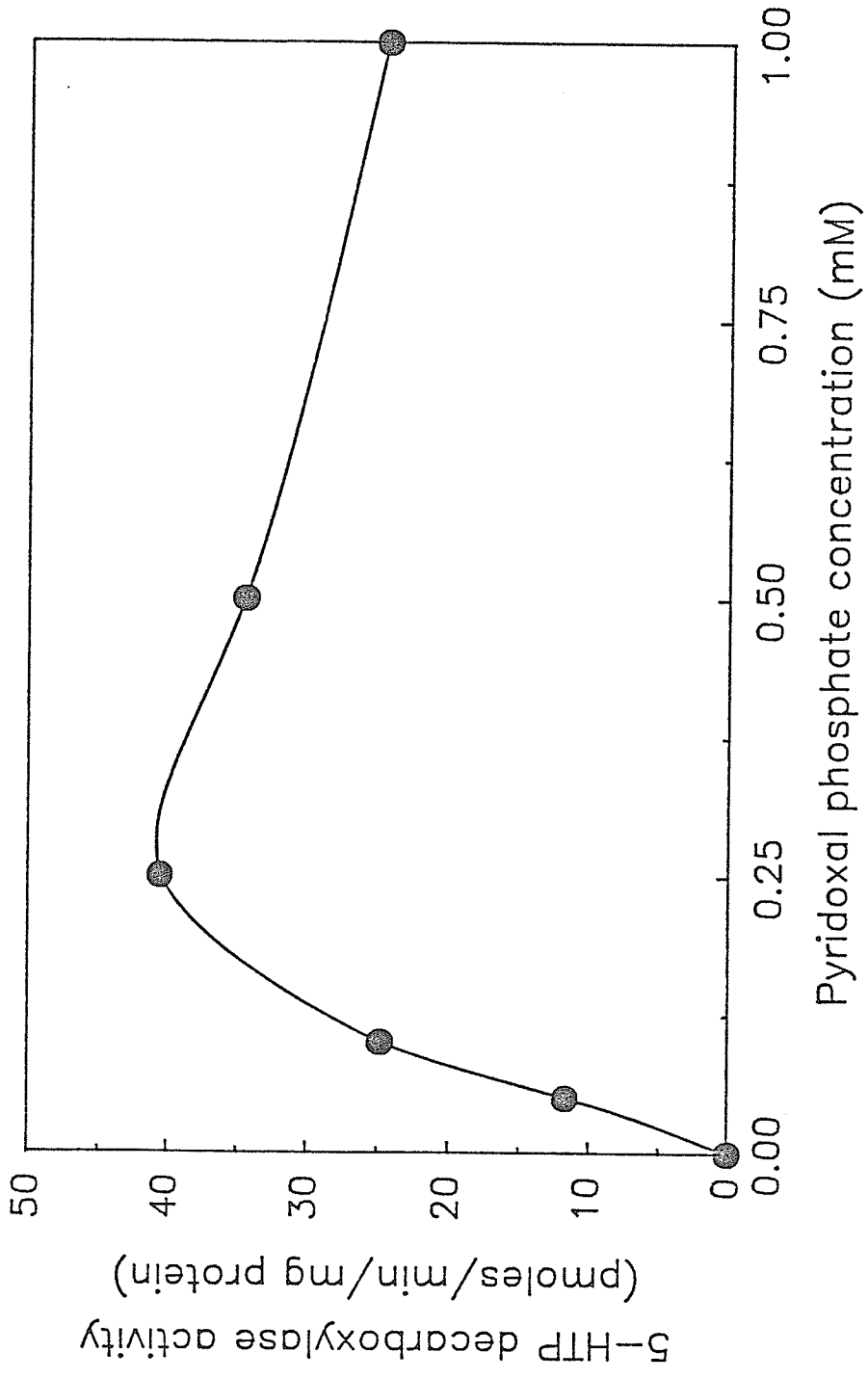

Figure 10. Effect of PLP on rat brain 5-HTP decarboxylase activity. 5-HTP decarboxylase activity was measured as described in Chapter III Section I (Part A).B.6, except that the concentration of PLP was varied as indicated.

TABLE 12

Regional distribution of DOPA decarboxylase and 5-HTP decarboxylase in the Rat Brain

Brain regions	DOPA decarboxylase[a]	5-HTP decarboxylase[a]	Ratio[a,b,c]
Brainstem	902±72 (5)	82.6±8.0 (5)	11.2±1.1* (5)
Cerebellum	754±52 (6)	28.2±4.1 (6)	30.0±5.2† (6)
Cerebral cortex	1596±160 (6)	40.9±5.1 (6)	40.2±3.9† (6)
Corpus Striatum	3474±343 (5)	221.4±17.8 (5)	16.5±2.6* (5)
Hypothalamus	3693±544 (5)	100.8±7.2 (5)	37.3±6.6† (5)

[a]Values represent mean±S.E.M. of (n) separate experiments, each performed in duplicate. Enzyme activities are expressed in pmol/min/mg protein.

[b]Ratio = $\frac{\text{DOPA decarboxylase activity}}{\text{5-HTP decarboxylase activity}}$

[c]Values are calculated from the two decarboxylation activities of individual samples. Ratios having the same superscript symbols are not significantly different from each other while those having different superscript symbols are significantly different from other values in the same column; $p<0.05$ Duncan's multiple range test.

with the pyridoxine-supplemented rats (Table 13). Similar results were observed when DOPA decarboxylase was assayed without the *in vitro* addition of PLP. Even if the maximal enzyme activity between the pyridoxine-deficient and pyridoxine-supplemented rat hypothalami were different, these results does not alter the general interpretation of the data as other brain regions such as brainstem, cerebellum, cerebral cortex, and corpus striatum do have significant differences in enzyme activity between the pyridoxine-deficient and pyridoxine-supplemented groups. However, the significant fact is that of the various tissues, only in the cerebellum was there a statistically significant decrease in the percent saturation of enzyme with PLP. No significant difference in percent saturation was observed in the cerebral cortex, corpus striatum and hypothalamus between the two groups (Table 13). The differences in the maximal and holoenzyme activities can be attributed to the regional difference in PLP levels in the brain as it has been reported earlier that PLP levels was decreased by 70% in the brainstem and 53% in the cerebellum of the pyridoxine-deficient rats (Singer, 1981). It should be noted that even when the activity of the enzyme decarboxylating DOPA is decreased in the deficient rat to about 29.5% of the pyridoxine-supplemented rat, it does not result in a decrease in the dopamine content in different brain regions of the deficient rat. This is due to the high DOPA decarboxylase activity in the brain.

When the 5-HTP decarboxylase was assayed under optimal substrate and PLP concentration, a significant decrease in activity was observed in the brainstem ($p<0.005$), cerebellum ($p<0.005$), cerebral cortex ($p<0.025$), corpus striatum ($p<0.005$) and the hypothalamus ($p<0.001$) of

TABLE 13

Comparison of DOPA decarboxylase activity in brain regions of pyridoxine-deficient ($-B_6$) and pyridoxine-supplemented ($+B_6$) rats[a]

	Maximal enzyme activity (With added PLP)		Holoenzyme activity (No added PLP)		%Saturation (Holo/Maximal x 100)	
Brain Regions	$-B_6$	$+B_6$	$-B_6$	$+B_6$	$-B_6$	$+B_6$
Brainstem	248± 36[b,c]	903± 72	<83[f]	<83[f]	<33.5%	<9.2%
Cerebellum	433±117[e]	806± 67	146± 45[c]	617± 43	37.0± 6.6[d]	77.6±5.2
Cerebral Cortex	595± 41[c]	1822± 70	426± 43[c]	1162±119	71.6± 5.3	63.9±6.0
Corpus striatum	1992±222[c]	3932±169	534±157[d]	1463±106	26.4± 7.4	37.3±2.5
Hypothalamus	2520±413	3585±481	1459±428	2121±509	54.0±10.0	56.3±7.7

[a]Values represent mean±S.E.M. of 5 separate experiments ([b]n=4), each performed in duplicate. Enzyme activities are expressed in pmol/min/mg protein.

[c]P<0.001 [d]P<0.005 [e]P<0.025 compared to pyridoxine-supplemented (Student's unpaired t-test).

[f]Limit of detection was 83 pmol/min/mg protein.

the pyridoxine-deficient rats as compared to the pyridoxine-supplemented rats (Table 14). A significant decrease in the holoenzyme species and percent saturation of enzyme with PLP was also observed in all the brain regions studied in the deficient animals.

The percent reductions of maximal and holoenzyme activities of both DOPA and 5-HTP decarboxylase in various brain regions of the pyridoxine-deficient rats as compared to that of the supplemented group are given in Table 15. The extent of the decreases were significantly different for the two decarboxylases. This cannot be ascribed to changes in the amino acid substrate as the enzymes were assayed under optimal amino acid substrate concentration. Furthermore, the decrease in PLP levels seen in various regions of the pyridoxine-deficient rats (Singer, 1981) cannot account for the non-identical changes in the holoenzyme activities of both DOPA decarboxylase and 5-HTP decarboxylase (Table 15).

5. Affinity of DOPA and 5-HTP decarboxylases for PLP

It has been reported that the dialysis of human serum with buffer containing hydroxylamine resulted in a fall in PLP content to less than 1 ng/ml (Lipson *et al.*, 1980). This method has been adapted for use with brain homogenates in this study. When the homogenate was dialyzed against buffer containing hydroxylamine, a decrease in DOPA decarboxylase activity was observed (Table 16). In contrast, a total loss in 5-HTP decarboxylase activity was observed in homogenates treated the same way. With the *in vitro* addition of optimal PLP concentration,

TABLE 14

Comparison of 5-HTP decarboxylase activity in brain regions of pyridoxine-deficient ($-B_6$) and pyridoxine-supplemented ($+B_6$) rats[a]

	Maximal enzyme activity (With added PLP)		Holoenzyme activity (No added PLP)		%Saturation (Holo/Maximal x 100)	
Brain Regions	$-B_6$	$+B_6$	$-B_6$	$+B_6$	$-B_6$	$+B_6$
Brainstem	35.6± 8.3[c]	82.6± 8.1	4.8±1.8[c]	32.4±11.6	11.4±5.1[e]	48.9±15.1
Cerebellum	4.8± 1.2[c]	13.9± 1.4	<0.6[f]	0.7± 0.6	<12.5	5.3± 4.9
Cerebral Cortex	17.8± 2.5[e]	37.9± 6.1	<0.6[f]	1.2± 1.1	<3.4	3.5± 2.9
Corpus striatum	133.3±24.4[c]	267.9±17.3	18.6±3.9[b]	89.6± 9.2	15.8±4.4[d]	33.3± 2.2
Hypothalamus	49.7± 3.4[b]	100.8± 7.2	2.1±1.3[c]	16.3± 3.3	4.5±3.0[e]	15.8± 2.2

[a]Values represent mean±S.E.M. of 5 experiments, each performed in duplicate. Enzyme activities are expressed in pmol/min/mg protein.

[b]$P<0.001$ [c]$P<0.005$ [d]$P<0.01$ [e]$P<0.025$ compared to pyridoxine-supplemented (Student's unpaired t-test).

[f]Limit of detection was 0.6 pmol/min/mg protein.

TABLE 15

Distribution of DOPA decarboxylase and 5-HTP decarboxylase Activities in Pyridoxine-deficient Rat Brain Regions relative to that in Pyridoxine-supplemented (normal) Rats

Brain regions	Maximal enzyme activity of deficient rat brain as percent of normals[a]		Holoenzyme activity of deficient rat brain as percent of normals[a]	
	DOPA decarboxylase	5-HTP decarboxylase	DOPA decarboxylase	5-HTP decarboxylase
Brainstem	29.5±1.1[d]	43.1±4.2	-[g]	14.5±0.4
Cerebellum	52.5±2.0[e]	32.7±1.9	26.3±7.8	0
Cerebral cortex	30.6±1.2[c]	40.9±1.4	31.5±4.2	0
Corpus striatum	55.9±2.3[f]	43.1±3.6	39.2±4.4[f]	23.9±0.6
Hypothalamus	73.3±1.4[b]	48.7±0.7	61.7±2.1[d]	16.4±8.0

[a]Percentages represent mean±S.E.M. calculated from the data of Tables 13 and 14; [b]$p<0.001$, [c]$p<0.005$, [d]$p<0.01$, [e]$p<0.025$ [f]$p<0.05$ compared to the respective 5-HTP decarboxylase activity (Student's unpaired t-test).

[g]There is no detectable holoenzyme activity in both the pyridoxine-deficient and pyridoxine-supplemented rats.

TABLE 16

Effect of Dialysis on Whole Brain
DOPA decarboxylase and 5-HTP decarboxylase

Conditions	PLP added (nM)	DOPA decarboxylase (Percent activity)[a]	5-HTP decarboxylase (Percent activity)[a]
Undialyzed whole brain homogenate	0	26	8
	25	89	-
	50	92	-
	125	100[b]	64
	300	-	100[c]
Homogenate dialyzed against buffer	0	31	0
	25	101	-
	50	92	-
	125	93	45
	300	-	103
Homogenate dialyzed against buffer containing hydroxylamine	0	26	0
	25	27	-
	50	36	-
	125	65	0
	150	-	14
	300	-	69

[a]Values represent the average of 3 separate experiments with a standard error of less than 5%.

[b]Represents an activity of 1024 pmol/min/mg protein.

[c]Represents an activity of 39.2 pmol/min/mg protein.

65% of DOPA decarboxylase activity and 69% of 5-HTP decarboxylase activity, respectively, were present in the dialyzed homogenates.

When brain homogenate was dialyzed against the buffer without hydroxylamine, there was no change in DOPA decarboxylase activity (Table 16). In contrast, there was a total loss of 5-HTP decarboxylase activity. Following *in vitro* addition of PLP, enzyme activities were restored.

D. Discussion

Pyridoxal phosphate is the coenzyme of the decarboxylases involved in the formation of various monoamine neurotransmitters. The enzyme involved in the decarboxylation of DOPA to DA and 5-HTP to 5-HT has been referred to as aromatic L-amino acid decarboxylase (Lovenberg *et al.*, 1962). In examining the decarboxylations of DOPA and 5-HTP in normal brain homogenates, it was found that the pH optima for these two reactions were different. Differences in the regional distribution of DOPA decarboxylase and 5-HTP decarboxylase activities in various tissues including the brain have been reported (Sims *et al.*, 1973; Rahman *et al.*, 1981a,b; Rahman and Nagatsu, 1982). The ratios of DOPA decarboxylase/5-HTP decarboxylase activities under optimal substrate and cofactor concentrations were found to vary from 11.2 in the brainstem to 40.2 in the cerebral cortex in the normal rat (Table 12). The differences in the activity of AADC toward DOPA and 5-HTP, respectively, could be due to differences in the pH and nature of the buffer used to extract the enzyme from the tissue as well as the pH optima of the decarboxylation reaction itself. If so, the ratio of the activities (DOPA decarboxylase/5-HTP decarboxylase) should be the same in all the tissues studied as the conditions for extraction and assay of enzyme activities were identical for all the regions studied. Yet, these ratios varied considerably from one brain region to another indicating the presence of different amounts of decarboxylating activities specific for each substrate.

Although there were significant differences in the maximal and holodecarboxylase activities towards DOPA in some of the brain regions studied - brainstem, cerebellum, cerebral cortex and corpus striatum - the actual contents of DA determined in all the brain regions studied in the deficient rat were not different from those seen in similar regions of normal rat brain (Dakshinamurti *et al.*, 1987). It is evident that the higher decarboxylation activity towards DOPA can maintain the levels of DA even though the enzyme protein levels were decreased by pyridoxine deficiency. It is interesting to note that the brainstem DOPA decarboxylase also have different properties compared to that of other regions. Firstly, the brainstem DOPA decarboxylase has a lower affinity for PLP since no holoenzyme activity was detected in both the pyridoxine-deficient and pyridoxine-supplemented rats. Preliminary evidence also indicates that DOPA decarboxylase from the brainstem also exhibited different kinetic characteristics from that of the corpus striatum [Brainstem: K_m for DOPA = 751 μM, V_{max} = 2.15 nmol/min/mg protein; Corpus striatum: K_m for DOPA = 366 μM, V_{max} = 3.00 nmol/min/mg protein].

The decreases in both the maximal and holo 5-HTP decarboxylase are reflected in the significant decreases in the 5-HT content of various brain regions in the pyridoxine-deficient rat (Dakshinamurti *et al.*, 1987). We have also reported that the sensitivity of serotonin S_1 and S_2 receptors are increased in membrane preparations from various regions of the pyridoxine-deficient rat brain (Paulose and Dakshinamurti, 1985). The physiological consequences of the decrease in 5-HT in the deficient rat brain has also been discussed (Dakshinamurti, 1982; Dakshinamurti

et al., 1985a,b; See also Chapter II.D.4). It is conceivable that the decrease in enzyme levels (maximal enzyme activity) reflects a reduced synthesis of the enzyme caused by pyridoxine deficiency, which has been reported to decrease the synthesis of ribosomal and messenger RNA (Montjar *et al.*, 1965). Pyridoxine deficiency did cause a decrease in both DOPA and 5-HTP decarboxylase activities. However, it should be noted that pyridoxine deficiency did not cause an identical decrease in maximal AADC activity towards DOPA and 5-HTP in the brain regions studied (Table 15). The results presented suggest the independent regulation of the two decarboxylation activities.

The affinity of PLP for the different apodecarboxylases could vary. In the pyridoxine-deficient animal, the activity of the decarboxylase with the more tightly bound coenzyme would be higher than those with lower affinity between the apoenzyme and the coenzyme. This is supported by the observation in this study that DOPA decarboxylase activity is consistently higher than 5-HTP decarboxylase activity in the rat brain. DOPA decarboxylase also has a lower K_m for PLP in comparison to 5-HTP decarboxylase, indicating a higher affinity for the coenzyme. Additionally, in this study, dialysis of brain homogenate against buffer, in the presence or absence of hydroxylamine, resulted in a total or near total loss of 5-HTP decarboxylase activity as compared to that of DOPA decarboxylase. Hence, PLP seems to be more tightly bound to DOPA decarboxylase than to 5-HTP decarboxylase. Rahman and co-workers (1982) have studied the acute effects of PLP deficiency, produced by intraperitoneal injection of semicarbazide, on AADC activity with DOPA and 5-HTP as substrates. AADC activities toward both substrates were

decreased. However, after *in vitro* addition of PLP, the recovery patterns of the enzyme activities toward DOPA and 5-HTP were not parallel, suggesting the possibility of the presence of two different AADC with different affinities for DOPA and 5-HTP. Differences in the kinetic characteristics or other properties of enzymes catalyzing the same reaction in different species or even in different organs of the same species are known to exist. For example, glutamic acid decarboxylase is present in both neural and non-neural tissues. In contrast to the brain enzyme which is inhibited by anions and carbonyl-trapping agents (Chase and Walters, 1976) and stimulated by PLP, the non-neural glutamic acid decarboxylase requires high concentration of anions for maximal activity, is activated by carbonyl-trapping agents and is unaffected by added PLP (Haber *et al.*, 1970; See also Chapter II.C). Furthermore, tyrosine hydroxylase from bovine caudate nucleus has been reported to have a similar molecular weight but different kinetic properties compared with the enzyme from the bovine adrenal medulla (Oka *et al.*, 1982). The biochemical similarities between tyrosine hydroxylase and tryptophan hydroxylase, which go far beyond their substrate requirements, have also been described (Lovenberg and Victor, 1974; Kuhn and Lovenberg, 1983). It is possible that such similarities may exist for the decarboxylating activities of AADC for the substrates DOPA and 5-HTP. Using electrophoretic analysis, the decarboxylase from rat brain has been shown to exist in various polymeric forms (Cavalli-Sfoza *et al.*, 1974). It is possible that different polymeric forms of AADC have different enzyme properties.

Confirmation of the above notion awaits the isolation of AADC from the brain.

The data presented suggest that the effect of pyridoxine deficiency is not identical on the activity of AADC using DOPA and 5-HTP as substrates.

CHAPTER III

EXPERIMENTAL SECTION I (PART B)

EFFECT OF NEUROTOXINS, DECARBOXYLASE INHIBITORS, AND ANTIPYRIDOXINE COMPOUNDS ON AROMATIC L-AMINO ACID DECARBOXYLASE

Effect of the neurotoxins (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and 1-methyl-4-phenylpyridinium), decarboxylase inhibitors, and antipyridoxine compounds, on aromatic L-amino acid decarboxylase in rat brain.

A. Introduction

The appearance of the compound 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) as a contaminant of a synthetic meperidine analog in Northern California has left in its wake a group of young drug addicts suffering from a severe and irreversible form of Parkinson's disease (Davis *et al.*, 1979; Langston *et al.*, 1983; Ballard *et al.*, 1985). These patients have exhibited all the features of idiopathic parkinsonism and respond to standard medical therapy. Since then, the neurotoxic effects of MPTP have been widely studied in monkeys (Burns *et al.*, 1983; Jenner *et al.*, 1984; Langston *et al.*, 1984a,b,c; Chiueh *et al.*, 1984a; Irwin and Langston, 1985; Cohen *et al.*, 1985), several rodent species (Steranka *et al.*, 1983; Chiueh *et al.*, 1984a; Wallace *et al.*, 1984; Heikkila *et al.*, 1984a), and also the cat (Schneider *et al.*, 1986). In addition to the reported effects on the dopaminergic nigrostriatal system, MPTP treatment has been reported to affect some non-nigrostriatal catecholamine systems in the mouse (Gupta *et al.*, 1984; Hallman *et al.*, 1985) and the monkey (Burns *et al.*, 1983; Chiueh *et al.*, 1985). Since this was the first major breakthrough in research on Parkinsonism, much attention has been focused on elucidating the mechanism of action of MPTP causing cell death. It has been shown that

MPTP is oxidized *in vitro* (Chiba *et al.*, 1984; Johannessen *et al.*, 1985b) and *in vivo* (Heikkila *et al.*, 1984b; Langston *et al.*, 1984c; Markey *et al.*, 1984) to 1-methyl-4-phenylpyridinium ion (MPP^+) by monoamine oxidase B (MAO-B), through the formation of the intermediate, 1-methyl-4-phenyl-2,3-dihydropyridinium cation ($MPDP^+$) (Chiba *et al.*, 1985) (Figure 11). The conversion of MPTP to MPP^+ can be blocked by the MAO-B inhibitors, deprenyl, nialamide, pargyline, tranylcypromine (Chiba *et al.*, 1984; Heikkila *et al.*, 1984b,1985b; Langston *et al.*, 1984c; Markey *et al.*, 1984; Cohen *et al.*, 1985; Fuller and Steranka, 1985; Irwin and Langston, 1985; Nakamura and Vincent, 1986), MD 240928 (Fuller and Hemrick-Luecke, 1985b), AGN-1133, AGN-1135 (Heikkila *et al.*, 1985a), and MDL 72145 (Kindt and Heikkila, 1986). The intraneuronal location for the conversion of MPTP to MPP^+ is still controversial. Serotonergic neuron has been suggested as the site for this conversion (Nakamura and Vincent, 1986) but another study excluded that possibility (Melamed *et al.*, 1986). MPP^+ is recognized by the dopamine (DA) uptake system and can thereby gain selective access to DA terminals (Javitch and Snyder, 1985). Several laboratories have then shown that blockade of this DA uptake system *in vivo*, with a variety of uptake inhibitors, provides protection from the toxic effects of MPTP in the mouse (Javitch *et al.*, 1985; Melamed *et al.*, 1985; Pileblad and Carlsson, 1985; Ricaurte *et al.*, 1985; Sundstrom and Jonsson, 1985). Once inside the neuron, the exact mechanism by which MPP^+ exerts its neurotoxic effects is still unknown. Reports have shown that MPP^+ is actively taken up by liver mitochondria (Ramsey *et al.*, 1986), induces oxidative stress (Johannessen *et al.*, 1986), causes inhibition of NADH-linked substrate

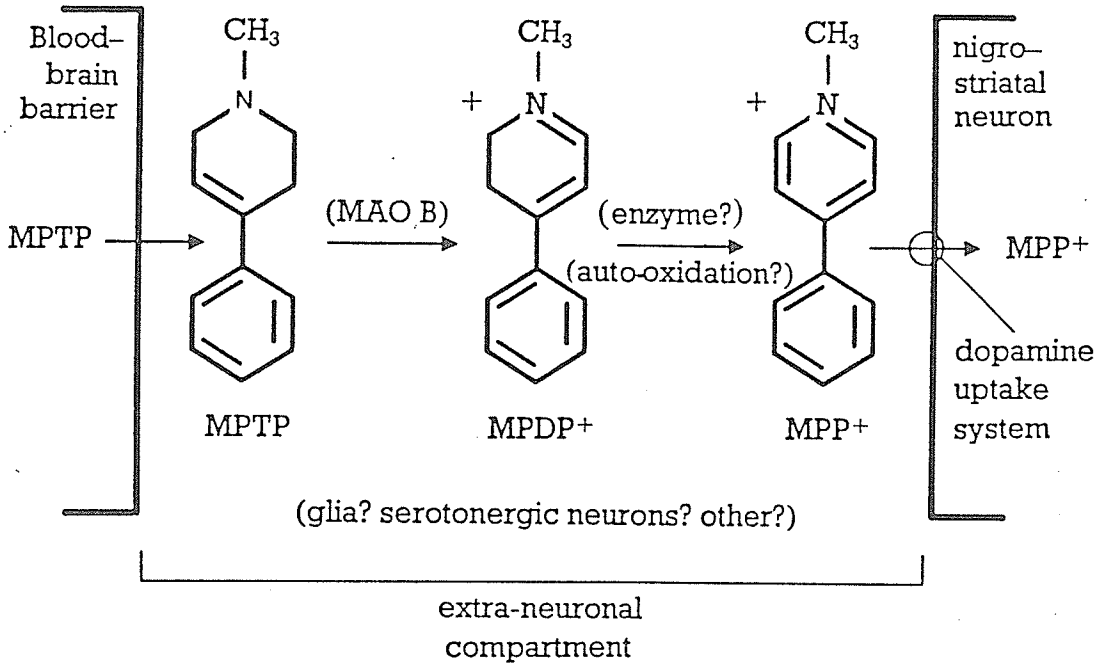

Figure 11. The metabolic fate of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine). MPTP enters the CNS where it is oxidized by monoamine oxidase B. The intermediate, $MPDP^+$ (1-methyl-4-phenyl-2,3-dihydropyridine) is then further oxidized to MPP^+ (1-methyl-4-phenylpyridinium) by a process not yet fully understood. MPP^+ is actively taken up by the dopamine uptake system into the nigrostriatal neurons causes various metabolic disturbances (Langston, 1985).

oxidation in brain mitochondria (Nicklas *et al.*, 1985), and rapidly depletes ATP in hepatocytes (DiMonte *et al.*, 1986). Studies also indicate that MPTP reduces DA concentration in rat brain (Chiueh *et al.*, 1984a; Enz *et al.*, 1984). In contrast, following *in vivo* administration of MPTP, brain serotonin (5-HT) level was either increased (Chiueh *et al.*, 1984a,b; Hallman *et al.*, 1985; Enz *et al.*, 1984; Tadano *et al.*, 1987) or unchanged (Hallman *et al.*, 1984; Heikkila *et al.*, 1984a; Wallace *et al.*, 1984; Jarvis and Wagner, 1985). However, the effect of MPTP on the enzyme catalyzing the formation of DA and 5-HT, aromatic L-amino acid decarboxylase (AADC, EC 4.1.1.28), has not been reported.

Many therapeutic agents also interfere with the normal function of vitamin B_6 both in man and laboratory animals (Ebadi *et al.*, 1982; Bhagavan and Brin, 1983; See also Chapter II.B.4.e: *Inhibitors*). For example, hydralazine (Perry *et al.*, 1955,1969; Schuler and Wyss, 1960), isonicotinic acid hydrazide (Killam and Bain, 1957; Wood and Abrahams, 1971), penicillamine (Giorgi and Rubio, 1981), and semicarbazides (Killam and Bain, 1957; Wood and Abrahams, 1971; Sawaya *et al.*, 1978) have been reported to prevent the formation of PLP or to inhibit PLP-dependent enzymes like DOPA decarboxylase and glutamate decarboxylase. As have been previously described (Chapter II.B.4.e: *Inhibitors*), decarboxylase inhibitors like α-methyl DOPA and the many hydrazides have been used to study the inhibition of AADC using either DOPA or 5-HTP as substrate. Reports of studies using both substrates have been limited to the use of carbidopa (Porter *et al.*, 1962; Borri Voltattorni and Minelli, 1977) and aminooxyacetic acid (AOAA) (Sims *et al.*, 1973).

The present study was undertaken to determine the *in vitro* effect of MPTP and its metabolite, MPP^+, on AADC using both 3,4-dihydroxyphenylalanine (DOPA) and 5-hydroxytryptophan (5-HTP) as substrates. We have also reexamined the *in vitro* effect of certain decarboxylase inhibitors and antipyridoxine metabolites on AADC using the two mentioned substrates.

B. Experimental

1. Materials

L-3,4-Dihydroxyphenyl[3-^{14}C]alanine (sp. act. 10.9 mCi/mmol) were purchased from Amersham Corporation (Oakville, ON). DL-5-[3-^{14}C]-hydroxytryptophan (sp. act. 59.0 mCi/mmol), β-[ethyl-1-^{14}C]-phenylethylamine hydrochloride (sp. act. 50.2 mCi/mmol), and [1-^{14}C]-tyramine hydrochloride (sp. act. 55.3 mCi/mmol) were obtained from New England Nuclear Corp. (Mississauga, ON). MPTP hydrochloride and MPP^+ were from Research Biochemicals Inc. (Wayland, MA). Pargyline (N-benzyl-N-methylpropargylamine) hydrochloride was from Saber Laboratories, Inc. (Morton Grove, IL) while NSD 1034 (N-(3-hydroxybenzyl)-N-methylhydrazine dihydrogen phosphate) and NSD 1055 (4-bromo-3-hydroxybenzyloxyamine dihydrogen phosphate; brocresin) were from Smith and Nephew (Essex, U.K.). α-Methyl DOPA, aminooxyacetic acid (AOAA) hydralazine hydro-chloride (Apresoline), isonicotinic acid hydrazide, NSD 1015 (*m*-hydroxybenzylhydrazine), penicillamine, phenelzine sulfate, semi-carbazide, and thiosemicarbazide were purchased from Sigma Chemical Co. (St. Louis, MO). Benserazide (Ro4-4602) was a gift from Hoffman-La Roche Inc. (Nutley, N.J.) while D,L-α-difluoromethyl DOPA (DFMD, MDL 71.801) and D,L-α-monofluoromethyl DOPA (MFMD, MDL 71.963) were gifts of Merell International (Strasbourg, France). Deprenyl was a kind gift from Professor J. Knoll, Department of Pharmacology, Semmelweis University of Medicine, Budapest, Hungary. All other chemicals were of reagent grade.

2. Tissue and sample preparation

Sprague-Dawley rats (150-200 g) were killed by decapitation, and their brains were rapidly removed, frozen on dry ice, and stored at -70°C. In assays using corpus striatum and hypothalamus, these brain regions were dissected according to Glowinski and Iversen (1966) prior to freezing and storage at -70°C. Tissue homogenates for decarboxylase assays were prepared as described in Chapter III Section I (Part A).B.3.

Crude brain mitochondria was prepared using the methods of Fowler and Tipton (1981) and Crane and Greenwood (1987) with modifications. Whole brain was homogenized in ten volumes of 0.32 M sucrose, 10 mM potassium phosphate, pH 7.2, using a Potter-Elvehjem homogenizer. The homogenates were centrifuged at 600 *g* for 10 min to remove unbroken cells, nuclei, and cell debris. The supernatant was carefully decanted and centrifuged at 15,000 *g* for 15 min. The mitochondrial pellet was resuspended in sucrose-phosphate buffer to a protein concentration of 5 mg/ml and stored frozen at -20°C until use.

3. Enzyme assays

DOPA decarboxylase and 5-HTP decarboxylase activities were assayed in whole brain homogenates as described previously in Chapter III Section I (Part A) B.5 and B.6, respectively, except that pargyline was excluded in the 5-HTP decarboxylase assay.

Monoamine oxidase (MAO) activity was assayed radiochemically by a modification of the method of Fowler and Tipton (1981). A mixture containing 200 µl 0.1 M potassium phosphate buffer, pH 7.2, 100 µl of mitochondrial preparation and 50 µl of water, MPTP, MPP^+, or pargyline,

was allowed to equilibrate with continuous shaking at 37°C for 30 min. Fifty microliters of tyramine (containing 0.02 μCi [1-^{14}C]-tyramine) was then added and a further incubation period of 30 min was carried out. The reaction was stopped by the addition of 100 μl of 2 M citric acid. Blank values were obtained by the addition of citric acid prior to addition of substrate. Five ml of OCS (Organic Counting Scintillant, Amersham) was then added to each mixture. This was vortexed and centrifuged and tubes were placed in a freezer for at least 3 hours to allow the aqueous layer to freeze. The organic layer was then poured out into a scintillation vial and the radioactivity was determined in a Beckman LS 2800 Liquid Scintillation System.

The B-form of MAO was assayed radiochemically by a modification of the method of Fuller and co-workers (1970, 1983). A mixture containing 200 μl 0.25 M sodium phosphate buffer, pH 7.4, 25 μl of mitochondrial preparation and 50 μl of water, MPTP, MPP^+, or deprenyl, was preincubated at 37°C for 5-10 min prior to addition of 25 μl of 12.5 uM phenylethylamine (containing 5 nCi of β-[ethyl-1-^{14}C]-phenylethylamine). After 10 min incubation at 37°C, the reaction was stopped by the addition of 0.2 ml of 2 M HCl. Heat-denatured mitochondrial preparation was used as blanks. The deaminated products were extracted into 6 ml toluene:ethyl acetate (1:1, v/v) containing 0.6% (w/v) of PPO (2,5-diphenyloxazole). The radioactivity of the organic layer was determined in a Beckman LS 2800 Liquid Scintillation System.

4. Measurement of protein

Protein concentration was measured using the Bio-Rad Protein Assay which is based on the method of Bradford (1976), with bovine serum albumin as a reference standard.

C. Results

1. Effect of the neurotoxins, MPTP and MPP+

The *in vitro* effects of MPTP and MPP^+ on the activities of DOPA decarboxylase and 5-HTP decarboxylase in rat brain are shown in Table 17. In the presence of 5 or 10 μM MPTP or MPP^+, the activity of 5-HTP decarboxylase increased by about 71-107%. In contrast, the activity of the DOPA decarboxylase was relatively unchanged. In addition, deprenyl at 0.1 μM and 0.5 μM did not alter the enhancement of 5-HTP decarboxylase activity seen in the presence of MPTP or MPP^+ (Table 18).

At 5 or 10 μM, MPTP caused a 13% and 20% inhibition, respectively, of MAO activity (Table 19). At similar concentrations, MPP^+ inhibited MAO activity by 27% and 39%, respectively. On the other hand, pargyline at 10 μM causes close to 100% inhibition of MAO activity. In the presence of 5 or 10 μM MPTP or MPP^+, MAO-B activity was not significantly altered (Table 19). Under the same assay conditions, deprenyl at substantially lower concentrations, significantly inhibited MAO-B activity.

2. Effect of the decarboxylase inhibitors

The effects of various decarboxylase inhibitors are shown in Table 20. At various inhibitor concentrations, both DOPA decarboxylase and 5-HTP decarboxylase activities were inhibited to similar extents by benserazide (Ro4-4602), MFMD, DFMD, NSD 1015, NSD 1034, and NSD 1055. However, this was not the case with α-methyl DOPA. A 17% and 46%

TABLE 17

Effects of MPTP and MPP^+ on Whole Brain DOPA decarboxylase and 5-HTP decarboxylase activities in the Rat

Compound	Conc. (uM)	DOPA decarboxylase activity[a,c]	5-HTP decarboxylase activity[b,c]
None		986±100	16.0±3.4
MPTP	5	1089±102 (+11%)[d]	27.3±6.0 (+71%)[e]
	10	1097±101 (+12%)[d]	29.6±6.0 (+86%)[e]
MPP^+	5	880±57 (-9%)[d]	31.6±8.0 (+96%)[e]
	10	909±53 (-6%)[d]	33.2±4.6 (+107%)[e]

[a]Brain homogenate was incubated at 37°C for 15 min in a medium (1-ml vol.) containing: 80 umoles sodium phosphate buffer (pH 6.7), 0.125 umole PLP, 10 umoles 2-mercaptoethanol, 1 umole DOPA (containing 0.1 uCi L-[3-^{14}C]-DOPA) with and without MPTP or MPP^+.

[b]Brain homogenate was incubated at 37°C for 60 min in a medium (1-ml vol.) containing: 75 umoles Tris-HCl buffer (pH 8.3), 0.3 umole PLP, 10 umoles 2-mercaptoethanol, 0.6 umole 5-HTP (containing 0.1 uCi DL-[3-^{14}C]-5-HTP) with and without MPTP or MPP^+.

[c]Values represent mean±S.E.M. for five separate experiments, each performed in duplicate. Enzyme activities are expressed in pmoles/min/mg protein. Numbers in parentheses show percent changes from control. [d]Not significant, [e]$p<0.05$ in comparison to control (Student's unpaired t-test).

TABLE 18

Effects of Deprenyl on Rat Whole Brain 5-HTP decarboxylase activity in the presence of 5 μM MPTP or MPP^+

Compound	[Deprenyl] (μM)	5-HTP decarboxylase (Percent activity)[a]
Control	0	100[b]
MPTP	0	170
	0.1	169
	0.5	206
MPP^+	0	184
	0.1	216
	0.5	222

[a]Brain homogenate was incubated at 37°C for 60 min in a medium (1-ml vol.) containing: 75 μmoles Tris-HCl buffer (pH 8.3), 0.3 μmole PLP, 10 μmoles 2-mercaptoethanol, 0.6 μmole 5-HTP (containing 0.1 μCi DL-[3-^{14}C]-5-HTP), and 5 nmoles of MPTP or MPP^+. For deprenyl studies, the assay medium was preincubated for 5 min at 37°C prior to the addition of 5-HTP. Values are expressed as percent of control and represent the average obtained for two separate experiments with a standard error of less than 5%.

[b]Represents an activity of 31.73 pmoles/min/mg protein.

TABLE 19

Effects of MPTP, MPP^+, Pargyline, and Deprenyl on MAO activity in crude Rat Brain Mitochondrial preparations

Compound	Conc. (μM)	MAO (Percent activity)[a]	MAO-B (Percent activity)[b]
None		100[c]	100[d]
MPTP	5	87±2	104
	10	80±2	94
MPP^+	5	73±1	117
	10	61±1	119
Pargyline	10	6±1	N.D.
Deprenyl	0.05	N.D.	33
	0.10	N.D.	16
	0.50	N.D.	13

[a]Crude brain mitochondrial preparation was incubated at 37°C for 30 min in a medium (0.4-ml vol.) containing: 20 μmoles potassium phosphate buffer (pH 7.2), MPTP, MPP^+ or pargyline as appropriate and 0.4 μmole tyramine (containing 0.02 μCi of [1-^{14}C]-tyramine). Values represent percent of control±S.E.M. for 5 separate experiments, each performed in duplicate; N.D.= not determined.

[b]Crude brain mitochondrial preparation was incubated at 37°C for 20 min in a medium (0.3-ml vol.) containing: 50 μmoles sodium phosphate buffer (pH 7.4), MPTP, MPP^+ or deprenyl as appropriate and 0.31 nmole tyramine (containing 5 nCi ß-[ethyl-1-^{14}C]phenylethylamine). Values are expressed as percent of control and represent the average obtained for two separate experiments with standard error less than 7% of the mean; N.D.= not determined.

[c]Represents an activity of 4.61±0.40 nmol/min/mg protein.

[d]Represents an activity of 1.37 nmol/min/mg protein.

TABLE 20

Effects of Decarboxylase Inhibitors on Striatal DOPA decarboxylase and Hypothalamic 5-HTP decarboxylase activities in the Rat

Compound	Conc. (μM)	DOPA decarboxylase (Percent Activity)[a]	5-HTP decarboxylase (Percent activity)[b]
Difluoromethyl-DOPA	5	51.0±11.2[f]	78.4±10.6
	50	13.3±2.5	11.8±0.9
Monofluoromethyl-DOPA	10	4.6±3.2[c]	13.4±2.8
	100	0	4.8±4.6
α-Methyl-DOPA	100	83.3±1.9[c]	44.9±0.3
	500	53.5±0.9[c]	16.8±0.6
NSD 1015	1	N.D.	28.2±2.4
	10	2.7±1.1[d]	19.9±5.9
	100	0.4±0.2	N.D.
NSD 1034	1	N.D.	38.0±8.8
	5	0	N.D.
	10	0	12.6±3.0
NSD 1055	1	N.D.	3.9±1.5
	10	0	5.7±0.6
	100	0	N.D.
Ro4-4602 (Benserazide)	10	7.2±5.5	15.2±0.2
	50	12.4±3.1[f]	0.4±0.2

[a]Striatal homogenate was incubated at 37°C for 15 min in a medium (1-ml vol.) containing: 80 μmoles sodium phosphate buffer (pH 6.7), 0.125 μmole PLP, 10 μmoles 2-mercaptoethanol, 1 μmole DOPA (containing 0.1 μCi L-[3-^{14}C]-DOPA) and the appropriate concentration of inhibitor; incubation mixture was preincubated for 5 min prior to the addition of DOPA. Values represent mean±S.D. (n=3) and are expressed as percent of control which has activities of 2541 - 3490 pmol/min/mg protein; N.D.= not determined.

[b]Hypothalamic homogenate was incubated at 37°C for 60 min in a medium (1-ml vol.) containing: 75 μmoles Tris-HCl buffer (pH 8.3), 0.3 μmole PLP, 10 μmoles 2-mercaptoethanol, 0.6 μmole 5-HTP (containing 0.1 μCi DL-[3-^{14}C]-5-HTP) and the appropriate concentration of inhibitor; incubation mixture was preincubated for 5 min prior to the addition of 5-HTP. Values represent mean±S.D. (n=3) and are expressed as percent of control which has activities of 136 - 178 pmol/min/mg protein; N.D.= not determined.

[c]$p<0.001$, [d]$p<0.01$, [e]$p<0.025$, [f]$p<0.05$ compared to respective 5-HTP decarboxylase activity (Student's unpaired t-test).

inhibition of DOPA decarboxylase activity was observed in the presence of 100 uM and 500 uM of -methyl DOPA. In contrast, 5-HTP decarboxylase activity was inhibited by 55% and 83%, respectively at those concentrations.

3. Effect of the antipyridoxine metabolites

Table 21 shows the effects of several antipyridoxine metabolites. Compounds like AOAA, hydralazine, and phenelzine differentially affected DOPA decarboxylase and 5-HTP decarboxylase activities. Other compounds - isonicotinic acid hydrazide (isoniazid), penicillamine, semicarbazide, thiosemicarbazide - did not significantly affect either enzyme activity.

TABLE 21

Effects of Anti-Pyridoxine Metabolites on Striatal DOPA decarboxylase and Hypothalamic 5-HTP decarboxylation activities in the Rat

Compound	Conc. (μM)	DOPA decarboxylase (Percent Activity)[a]	5-HTP decarboxylase (Percent activity)[b]
Aminooxyacetic acid	100	83.1±1.0[f]	90.8±4.6
	1000	16.5±1.4[e]	80.2±3.3
Hydralazine	100	89.4±2.8[f]	99.6±5.2
	500	21.5±7.0[e]	40.9±5.6
Isonicotinic acid hydrazide	100	123.0±0.4	119.7±8.8
	1000	104.5±0.6	105.4±0.3
Penicillamine	100	104.3±6.2	95.1±3.0
	1000	114.9±7.8[f]	98.9±6.0
Phenelzine	10	N.D.	89.6±2.6
	100	53.2±2.7[d]	77.8±1.9
Semicarbazide	100	103.9±8.6	112.4±5.2
	1000	105.9±5.6	105.2±1.1
Thiosemicarbazide	100	101.8±5.4	106.3±2.5
	1000	84.5±3.4	83.1±5.2

[a]Striatal homogenate was incubated at 37°C for 15 min in a medium (1-ml vol.) containing: 80 μmoles sodium phosphate buffer (pH 6.7), 0.125 μmole PLP, 10 μmoles 2-mercaptoethanol, 1 μmole DOPA (containing 0.1 μCi L-[3-^{14}C]-DOPA) and the appropriate concentration of metabolite; incubation mixture was preincubated for 5 min prior to the addition of DOPA. Values represent mean±S.D. (n=3) and are expressed as percent of control which has activities of 2856 - 3490 pmol/min/mg protein; N.D.= not determined.

[b]Hypothalamic homogenate was incubated at 37°C for 60 min in a medium (1-ml vol.) containing: 75 μmoles Tris-HCl buffer (pH 8.3), 0.3 μmole PLP, 10 μmoles 2-mercaptoethanol, 0.6 μmole 5-HTP (containing 0.1 μCi DL-[3-^{14}C]-5-HTP) and the appropriate concentration of metabolite; incubation mixture was preincubated for 5 min prior to the addition of 5-HTP. Values represent mean±S.D. (n=3) and are expressed as percent of control which has activities of 136 - 191 pmol/min/mg protein.

[c]$p<0.001$, [d]$p<0.005$, [e]$p<0.025$, [f]$p<0.05$ compared to respective 5-HTP decarboxylase activity (Student's unpaired t-test).

D. Discussion

The increase in 5-HTP decarboxylase activity caused by MPTP and MPP^+ is in accordance with the increase in the brain level of 5-HT following *in vivo* administration of MPTP (Chiueh *et al.*, 1984a,b; Hallman *et al.*, 1985; Enz *et al.*, 1984; Tadano *et al.*, 1987). The low concentrations of MPTP and MPP^+ used in this investigation are in the range expected following intravenous administration of MPTP and have been used in organotypic culture of embryonic rat mesencephalon (Langston *et al.*, 1984a; Mytilineou and Cohen, 1984; Cohen and Mytilineou, 1985).

It has been reported that MPTP and MPP^+ are potent inhibitors of both forms of MAO (Kinemuchi *et al.*, 1985; Singer *et al.*, 1985). It is possible that the increase in 5-HTP decarboxylase activity observed *in vitro* by MPTP or MPP^+ addition might be due to the inhibition of MAO. This would cause an accumulation of 5-HT. Thus, the increase in decarboxylase of 5-HTP observed might be an artifact of the assay conditions. If this were so, there should be an accumulation of both 5-HT and DA as well as an apparent increase in the activities of both DOPA and 5-HTP decarboxylases under the assay conditions. However, only 5-HTP decarboxylase activity was significantly increased, but no significant change in DOPA decarboxylase activity was observed. In addition, it was found that the inhibition of MAO by MPTP and MPP^+ at the two concentrations studied was only in the range of 13-40%. Thus, the inhibition of MAO alone cannot account for the increased 5-HTP decarboxylase activity. The selective inhibition of the two forms of

MAO by MPTP and MPP^+ (Kinemuchi *et al.*, 1985; Singer *et al.*, 1985) should not come into question since both DA and 5-HT can be deaminated by MAO-A in the rat brain (Finberg and Youdim, 1983; Fuller and Hemrick-Luecke, 1985a; Fowler and Ross, 1984; Arai *et al.*, 1986). The concentrations of MPTP and MPP^+ used also did not inhibit MAO-B activity (Table 20). Deprenyl, a specific MAO-B inhibitor, has been shown to prevent the oxidation of MPTP to MPP^+ (Chiba *et al.*, 1984; Heikkila *et al.*, 1984b,1985b; Cohen and Mytilineou, 1985; Cohen *et al.*, 1985; Fuller and Steranka, 1985; Nakamura and Vincent, 1986). When it was included in the assay for 5-HTP decarboxylase, the increase in decarboxylase caused by MPTP and MPP^+ remained unchanged (Table 19). Hence, the oxidative metabolism of MPTP to MPP^+ or the dihydropyridinium intermediate (Castagnoli *et al.*, 1985) is not required for the stimulation of 5-HTP decarboxylase.

The enzyme AADC has generally been referred to as a single enzyme capable of decarboxylating both DOPA and 5-HTP (Lovenberg *et al.*, 1962). However, earlier work described in Chapter III Section I (Part A) of this thesis has suggested the possible existence of different forms of AADC with different substrate specificities for DOPA and 5-HTP, respectively, being present in varying amounts in different brain regions. This would explain the differential effects of MPTP and MPP^+ on the activity of AADC using DOPA and 5-HTP as substrates in the rat brain. The increased 5-HT level (Chiueh *et al.*, 1984a,b; Enz *et al.*, 1984; Hallman *et al.*, 1985) seen in rats or mice administered MPTP could be explained by the action of MPTP and MPP^+ or their metabolites on 5-HTP decarboxylase. However, the mechanism of this action has yet to

be investigated. It should also be noted that it is possible that this action of MPTP and MPP^+ on 5-HTP decarboxylase may have no bearing on the nigral cell death induced by MPTP in mice (Heikkila *et al.*, 1984a; Hallman *et al.*, 1985; Gupta *et al.*, 1984) since DOPA decarboxylase was not affected. Furthermore when MPTP is administered in low concentrations to rats, it does not cause selective destruction of dopaminergic neurons (Chiueh *et al.*, 1984a,b; Enz *et al.*, 1984). However, there is considerable evidence indicating a functional relationship between 5-HT, NE and DA in the control of nigrostriatal function (Chesselet, 1984). The 5-HT innervation of the substantia nigra and caudate nucleus comes from the dorsal and median raphe nuclei, the innervation of the substantia nigra being particularly rich. Thus, a decrease in DA release from the substantia nigra, caused by MPTP neurotoxicity, may in turn increase 5-HT release from terminals projecting to the substantia nigra or caudate nucleus. An increase in 5-HT release may be accompanied by an increase in the synthesis of 5-HT since 5-HT cell bodies are not affected by MPTP treatment (Gupta *et al.*, 1984; Hara *et al.*, 1987). Alternatively, intact serotonergic fibers may also sprout in response to reduced presence of dopaminergic terminals in the nigrostriatal system (Gupta *et al.*, 1984). Since primates and rodents exhibit marked differences in their sensitivity to the neurotoxic effects of MPTP (Johannessen *et al.*, 1985a), it would be of interest to examine the effects of MPTP and MPP^+ on AADC in the primate brain.

The present study showed that the enzyme-activated irreversible inhibitors, DFMD and MFMD, potently inhibited both DOPA decarboxylase

and 5-HTP decarboxylase *in vitro*. When administered intraperitoneally, both DFMD (Palfreyman *et al.*, 1978) and MFMD (Jung *et al.*, 1979) inhibited AADC activity in many organs. However, only MFMD was an effective inhibitor of AADC activity in the brain (Jung *et al.*, 1979). This is probably due to the poor penetration of DFMD through the blood-brain barrier since it is shown in this study that DFMD is as potent as MFMD as an inhibitor of DOPA and 5-HTP decarboxylases in the brain. The antihypertensive agent, α-methyl DOPA has also been shown to inhibit DOPA decarboxylase (Sourkes, 1954) and 5-HTP decarboxylase (Lovenberg *et al.*, 1963). Using pig kidney cortex as the enzyme source, Sourkes (1954) reported that 0.1 mM and 0.5 mM of α-methyl DOPA inhibited 71% and 98% of DOPA decarboxylase activity, respectively. At similar concentrations, we found that rat striatal DOPA decarboxylase was inhibited to a lesser extent. This may be attributed to a difference in the tissue selectivity of the inhibitor or to a difference in the characteristics of DOPA decarboxylase from the CNS versus the peripheral organs. Furthermore, α-methyl DOPA was found to inhibit 5-HTP decarboxylase to a greater extent than DOPA decarboxylase. Comparable inhibition of 5-HTP decarboxylase activity was also observed by Lovenberg and colleagues (1963). Complete inhibition of DOPA decarboxylase by NSD 1015 and NSD 1055 (Giorgi and Rubio, 1981) was observed at 10 μM while NSD 1034 (2 μM) causes 50% inhibition (Sourkes and Rodriguez, 1967). It was observed in this study, all the NSD compounds - NSD 1015, NSD 1034, NSD 1055 - strongly inhibited the two decarboxylase activities with NSD 1055 being the most potent. Burkard and co-workers (1962,1964) reported that benserazide not only inhibited

rat kidney 5-HTP decarboxylase (70% at 50 nM) but also rat brain 5-HTP decarboxylase (50% at 10 μM). Other reports have shown that benserazide totally inhibited DOPA decarboxylase at 7-30 μM (Bartholini *et al.*, 1967; Giorgi and Rubio, 1981). In this study, we report that although there was inhibition of DOPA decarboxylase and 5-HTP decarboxylase by benserazide, the inhibition was not selective for either enzyme.

The present study also shows that the non-specific carbonyl-trapping agent, AOAA, inhibited DOPA decarboxylase to a greater extent than 5-HTP decarboxylase. This is in agreement with the observations of Sims and co-workers (1973). However, DOPA decarboxylase from the adrenals were not affected by AOAA *in vitro* or *in vivo* (Giorgi and Rubio, 1981). This again shows differences in the characteristics of DOPA decarboxylase from the CNS and the peripheral organs. The antihypertensive agent, hydralazine (Apresoline), has also been reported to inhibit DOPA decarboxylase in the kidney (Perry *et al.*, 1955, 1969). When included in enzyme assays in this study, hydralazine is shown to inhibit both DOPA and 5-HTP decarboxylase activities with a more pronounced effect on the former enzyme. Isonicotinic acid hydrazide, commonly known as INH, has been extensively used as an antituberculous drug (Ebadi *et al.*, 1982; Bhagavan and Brin, 1983). This hydrazino compound reacts with PLP, forming a nicotinyl hydrazone of pyridoxal, which is excreted in urine (Ebadi *et al.*, 1982; Bhagavan and Brin, 1983). This depletes the tissue of PLP and thus may act as a competitive inhibitor of PLP-dependent enzymes. This was true for GAD where 32-57% inhibition was observed in the presence of 0.1-1.5 mM INH (Killam and Bain, 1957; Wood and Abrahams, 1971). In the present study,

not only did INH not cause any inhibition of DOPA decarboxylase or 5-HTP decarboxylase activities, there was a slight increase in the two decarboxylase activities at 0.1 mM. Nevertheless, this effect was not selective for either enzyme. The *in vivo* effects of various antivitamin B_6 like penicillamine, semicarbazide, and thiosemicarbazide on the metabolism of γ-aminobutyric acid (GABA) and its relationship to vitamin B_6 has been well studied (Killam and Bain, 1957; Wood and Abrahams, 1971; Fisher and Davies, 1974; Perry *et al.*, 1974; Yamashita and Hirata, 1977; Sawaya *et al.*, 1978; Abe and Matsuda, 1979; Sakurai *et al.*, 1981). These compounds are known to produce seizures in animals and the above studies concur that the interaction of these compounds with PLP causes an inhibition of glutamic acid decarboxylase (GAD), leading to a decrease in the levels of GABA in the brain; GAD requires PLP as its coenzyme in the decarboxylation of glutamate to GABA. The deficiency of GABA in the brain is an important factor in the production of convulsions. Although hydrazides like semicarbazide and thiosemicarbazide strongly inhibited GAD activity at 0.1-1 mM concentrations (Killam and Bain, 1957; Wood and Abrahams, 1971; Fisher and Davies, 1974), these compounds were found to be ineffective in inhibiting DOPA decarboxylase or 5-HTP decarboxylase. Penicillamine is a metal-chelating agent and has been used in the treatment of Wilson's disease to reduce the copper load in patients (Bhagavan and Brin, 1983). This agent has also been used in the treatment of arthritis (Ebadi *et al.*, 1982). This compound was reported to inhibit GAD activity *in vitro* and *in vivo* (Abe and Matsuda, 1979; Giorgi and Rubio, 1981) while DOPA decarboxylase activity was increased *in vitro* (Giorgi and Rubio,

1981). The present study shows no significant effect of penicillamine on both decarboxylase activities. Phenelzine has been shown to cause clinical pyridoxine deficiency (Stewart *et al.*, 1984; Demers *et al.*, 1984). The probable mechanism of induction of pyridoxine deficiency is a combination of the hydrazine ($-NHNH_2$) moiety with the pyridoxal form of the vitamin to produce a hydrazone, which is inactive (Raskin and Fishman, 1964). Phenelzine is also a non-specific monoamine oxidase inhibitor and has been used for treatment of phobic states, atypical depressions, and posttraumatic stress disorders (Demers *et al.*, 1984). It has been shown to inhibit brain 5-HTP decarboxylase as well (Dyck and Dewar, 1986). The current study indicates that phenelzine seems to have a more pronounced effect on DOPA decarboxylase activity than 5-HTP decarboxylase activity.

In summary, our results show that MPTP and MPP^+ have differential effects on the activity of AADC, using DOPA and 5-HTP, respectively, as substrates in the rat brain. The oxidative metabolism of MPTP and MPP^+ is not essential for the stimulation of 5-HTP decarboxylase activity. Furthermore, α-methyl DOPA, AOAA, hydralazine, and phenelzine also show differential effects on the decarboxylase activities.

CHAPTER III

EXPERIMENTAL SECTION II

PURIFICATION OF DOPA DECARBOXYLASE FROM BOVINE STRIATUM

Purification of DOPA decarboxylase from bovine striatum

A. Introduction

The experimental results of the previous chapters suggest the independent regulation of the decarboxylations of DOPA and 5-HTP. This regulation may involve one or more forms of decarboxylase for aromatic amino acids in the brain. To confirm the identity of AADC in the brain, it is necessary to purify the protein(s) in question.

Aromatic L-amino acid decarboxylase has been purified from various tissues such as guinea pig, pig, and rat kidneys (Christenson *et al.*, 1970; Lancaster and Sourkes, 1972; Srinivasan and Awapara, 1978; Borri Voltattorni *et al.*, 1979; Shirota and Fujisawa, 1988), bovine adrenals (Ceasar *et al.*, 1970; Goldstein, 1972; Albert *et al.*, 1987), rat liver (Awapara *et al.*, 1962; Ando-Yamamoto *et al.*, 1987; Dominici *et al.*, 1987), human pheochromocytoma (Maneckjee and Baylin, 1983; Ichinose *et al.*, 1985) and small intestine of monkey (Murali and Radhakrishnan, 1970). Recently, the purification of AADC from bovine brain stem, by affinity chromatography using a monoclonal antibody to the adrenal enzyme, has been reported by Nishigaki *et al.* (1988).

We now report for the first time the isolation and characterization of DOPA decarboxylase from bovine corpus striatum, a dopaminergic-rich source. We have also examined the regional distribution of AADC towards DOPA and 5-HTP in the bovine brain.

B. Experimental

1. Materials

DEAE-Sephacel and Sephacryl S-200 were from Pharmacia-LKB (Montreal, PQ). LKB-UltroPac column of TSK Phenyl 5PW was purchased from Fisher Scientific (Edmonton, AB). L-3,4-Dihydroxyphenyl[3-^{14}C]-alanine (11 mCi/mmol) and DL-5-hydroxy[G-^{3}H]tryptophan (5.3 Ci/mmol) were purchased from Amersham Canada (Oakville, ON). All other chemicals were of the highest grade commercially available.

Fresh bovine brains were obtained from a local abbatoir (East-West Packers Ltd., Winnipeg, MB) and transported on ice to the laboratory where different brain regions were quickly dissected out and kept frozen at -70^{o}C until use.

2. Enzyme assays

DOPA decarboxylase and 5-HTP decarboxylase activities were assayed as described previously in Chapter III Section I (Part A) B.5 and B.6, respectively. For the determination of DOPA decarboxylase activity of column eluates, a microassay using a smaller volume of sample was employed. The reaction mixture contained in a total volume of 0.1 ml: 80 mM sodium phosphate buffer (pH 6.7), 1 mM [^{14}C]DOPA (0.2 uCi/umol), 10 mM 2-mercaptoethanol, 0.125 mM pyridoxal 5'-phosphate, and 0.1 mM pargyline. The reaction was initiated by the addition of [^{14}C]DOPA solution and after incubation at 37^{o}C for 60 min, it was stopped by chilling on ice. The reaction mixture was extracted twice with 1-ml

volume of 1-butanol. Scintillant was added to the butanol fractions and the radioactivity determined.

3. Definition of enzyme unit

One enzyme unit is defined as the amount which catalyzes the formation of 1 nmol of dopamine per min at 37°C from L-DOPA. The specific activity is expressed as units/mg protein.

4. Determination of protein

Protein contents were determined using Bio-Rad Protein Assay which is based on the method of Bradford (1976) with bovine serum albumin as a reference standard, or by measuring the absorbance at 280 nm.

5. Polyacrylamide gel electrophoresis at pH 8.8

The degree of homogeneity of the purified bovine striatal DOPA decarboxylase was assessed by using either 7.5% SDS-PAGE or 7.5% non-SDS-PAGE at pH 8.8, followed by localization of proteins by silver staining. The SDS-PAGE system used was that of Laemmli (1970) while non-SDS-PAGE was as described by Davis (1964), using 0.75 mm thick slab gels (8 x 7 cm). After electrophoresis the gels were fixed for 30 min with 12.5% trichloroacetic acid and then silver stained using the system of Morrissey (1981). Relative mobilities of protein bands were calculated with respect to the following markers: phosphorylase *b* (M_r=97,400), bovine serum albumin (M_r=66,200), ovalbumin (M_r=42,699), carbonic anhydrase (M_r=31,000).

6. Purification of bovine striatal DOPA decarboxylase

It is important to work as quickly as possible in the cold at all stages of purification. All procedures described here were performed at $4^{o}C$ unless otherwise stated.

(a) *Preparation of the crude striatal extract*

Bovine striatum (about 300g) was homogenized in four volumes of 0.1 M potassium phosphate buffer, pH 6.8 in a Waring blender. The homogenate was then centrifuged at 10 000 *g* for 1 h in a Sorvall RC-5C refrigerated centrifuge. The supernatant was considered the crude extract.

(b) *Ammonium Sulfate Fractionation*

Crystalline ammonium sulfate was added to a gently stirring crude extract until 30% saturation was reached. The mixture was then left stirring for at least 30 min after which the precipitate was removed by centrifugation at 10 000 *g* for 1 h and discarded. To the supernatant was added additional solid ammonium sulfate until 60% saturation was reached. This was stirred gently for an additional 45 min, and then centrifuged at 13 000 *g* for 1 h and the supernatant was discarded. This 30-60% ammonium sulfate precipitate was slowly dissolved in 0.01 M potassium phosphate buffer, pH 7.8, containing 0.01 mM PLP, 0.1 mM EDTA and 1 mM 2-mercaptoethanol (Buffer A) and was dialyzed overnight against several changes of the same buffer. The dialyzed solution was centrifuged at 100 000 *g* for 1 h and the pellet was discarded.

(c) *DEAE-Sephacel column chromatography*

The centrifuged dialysate from the previous step was loaded at a flow rate of 35 ml/h onto a DEAE-Sephacel column (2.5 x 48 cm) previously equilibrated with Buffer A minus PLP and EDTA. The column was washed with 1 column volume of the same buffer (300 ml) followed by a linear gradient of 0.02 to 0.3 M potassium phosphate buffer (1 liter each), pH 7.8, containing 1 mM 2-mercaptoethanol. Fifteen milliliter fractions were collected and those with the highest enzyme activity were pooled. Solid ammonium sulfate was added to the pooled fractions to 60% saturation. The precipitate was then collected by centrifugation, dissolved in about 9 ml of 0.1 M potassium phosphate buffer, pH 6.8, containing 0.01 mM PLP, 0.1 mM EDTA and 1 mM 2-mercaptoethanol, and dialyzed overnight against the same buffer.

(d) *Sephacryl S-200 column chromatography*

Four milliliters of the dialysate obtained from the previous step was applied onto a Sephacryl S-200 gel filtration column (2.5 x 110 cm), previously equilibrated with 0.1 M potassium phosphate buffer, pH 6.8, containing 0.02 mM PLP, 0.1 mM EDTA and 2 mM 2-mercaptoethanol (Buffer B). The same buffer was used for elution at a flow rate of 14 ml/h and 2-ml fractions were collected. The gel filtration procedure was repeated a few times and samples with the highest enzyme activity were pooled and concentrated with an Amicon ultrafiltration unit using YM10 membrane filter.

(e) *Hydrophobic HPLC with Phenyl-5PW*

Hydrophobic chromatography was done on a Waters HPLC system (Milford, MA) consisting of the following components: Model 720 system controller, two solvent delivery systems (Model 45 & Model 6000A), Model U6K universal injector, and Model 411 absorbance detector. A UltroPac column of TSK Phenyl-5PW (i.d. 7.5 x 75 mm) was used for chromatography. To the enzyme solution solid ammonium sulfate was added to 15% saturation. The mixture was then applied to the hydrophobic column, previously equilibrated with a buffer containing 100 mM potassium phosphate buffer (pH 6.8), 15% ammonium sulfate, 0.1 mM PLP, 0.1 mM EDTA and 1 mM dithiothreitol (DTT). Then the column was washed with Buffer C consisting of 50 mM potassium phosphate buffer (pH 6.8), 0.1 mM PLP, 0.1 mM EDTA, 1 mM DTT, and 10% ammonium sulfate. Elution was carried out by a linear gradient of 10-0% ammonium sulfate in Buffer C at a flow rate of 0.5 ml/min.

7. Effects of sulfhydryl reagents and metal cations

Partially purified enzyme preparations (after DEAE-Sephacel chromatography) were dialyzed overnight in 0.1 M potassium phosphate buffer, pH 6.8, containing 0.01 mM PLP prior to determination of enzyme activity as described above. 2-Mercaptoethanol was omitted from the assay medium. The appropriate amounts of various sulfhydryl reagents and metal ion solutions were added to the assay medium and preincubated for 5 min and 2 min, respectively, at 37^{o}C prior to initiation of enzyme reaction.

To evaluate whether 2-mercaptoethanol and EDTA could reverse the effects of metal ions, enzyme solutions were preincubated as described above. After 2 min at 37°C, 1 mM 2-mercaptoethanol or 0.5 mM EDTA was added. The mixture was further incubated for 3 min at 37°C at which time the [^{14}C]DOPA substrate solution was added.

8. Effect of heat treatment

Aliquots of the same enzyme preparations (as in (f) above) were incubated at 55°C in the assay medium containing the buffer and 2-mercaptoethanol for various length of time. Pyridoxal 5'-phosphate was then added to the medium prior to the initiation of enzyme reaction by the addition of [^{14}C]DOPA substrate.

C. Results

1. Regional distribution of decarboxylase activities

The distribution of DOPA and 5-HTP decarboxylase activities was investigated in several brain regions. Expressed as a ratio of DOPA decarboxylase over 5-HTP decarboxylase activities (Table 22), it was found that the activity ratio varied from 8 in the brainstem and corpus striatum to 24 in the hypothalamus.

2. Purification of bovine striatal DOPA decarboxylase

DOPA decarboxylase from bovine striatum was purified using a combination of ammonium sulfate precipitation and ion-exchange (Figure 12), gel filtration (Figure 13), and hydrophobic interaction chromatography (Figure 14). The results from this purification protocol are summarized in Table 23. The ammonium sulfate fractionation at 30-60% saturation precipitated at least 80% of the enzyme activity while precipitating about 50% of the proteins in the crude extract. During DEAE-Sephacel chromatography, the decarboxylase appeared in the eluate when the phosphate-buffer concentration reached approximately 0.12 M (Fractions 68-76 in Figure 12). The specific activity of the final enzyme preparation with DOPA as substrate was about 1,600 units/mg protein with a yield of 6%.

3. Properties of bovine striatal DOPA decarboxylase

The purity of striatal DOPA decarboxylase was assessed by electrophoresis on polyacrylamide gels. One single band (M_r=56,000) was

TABLE 22

Regional Distribution of DOPA decarboxylase and 5-HTP decarboxylase in Bovine Brain

Brain regions	DOPA decarboxylase[a]	5-HTP decarboxylase[a]	Ratio[a,b,c]
Brainstem	392±22	48.2±11.8	9.0±1.9 *
Cerebellum	166±1	16.8±1.7	10.1±1.0 *
Cerebral cortex	291±82	17.0±0.2	17.0±4.6 †
Corpus Striatum	1381±127	169.1±3.1	8.1±0.6 *
Hippocampus	297±29	25.3±3.1	11.8±0.2 *,†
Hypothalamus	1478±7	62.4±1.1	23.7±0.3 ‡
Pineal Gland	5832±58	352.4±28	16.7±1.1 †

[a]Values represent mean±S.E.M. of 3 separate experiments. Enzyme activities are expressed in pmol/min/mg protein.

[b]Ratio = $\frac{\text{DOPA decarboxylase activity}}{\text{5-HTP decarboxylase activity}}$

[c]Values are calculated from the two decarboxylation activities of individual samples. Ratios having the same superscript symbols are not significantly different from each other while those having different superscript symbols are significantly different from other values in the same column; $p<0.05$ Duncan's multiple range test.

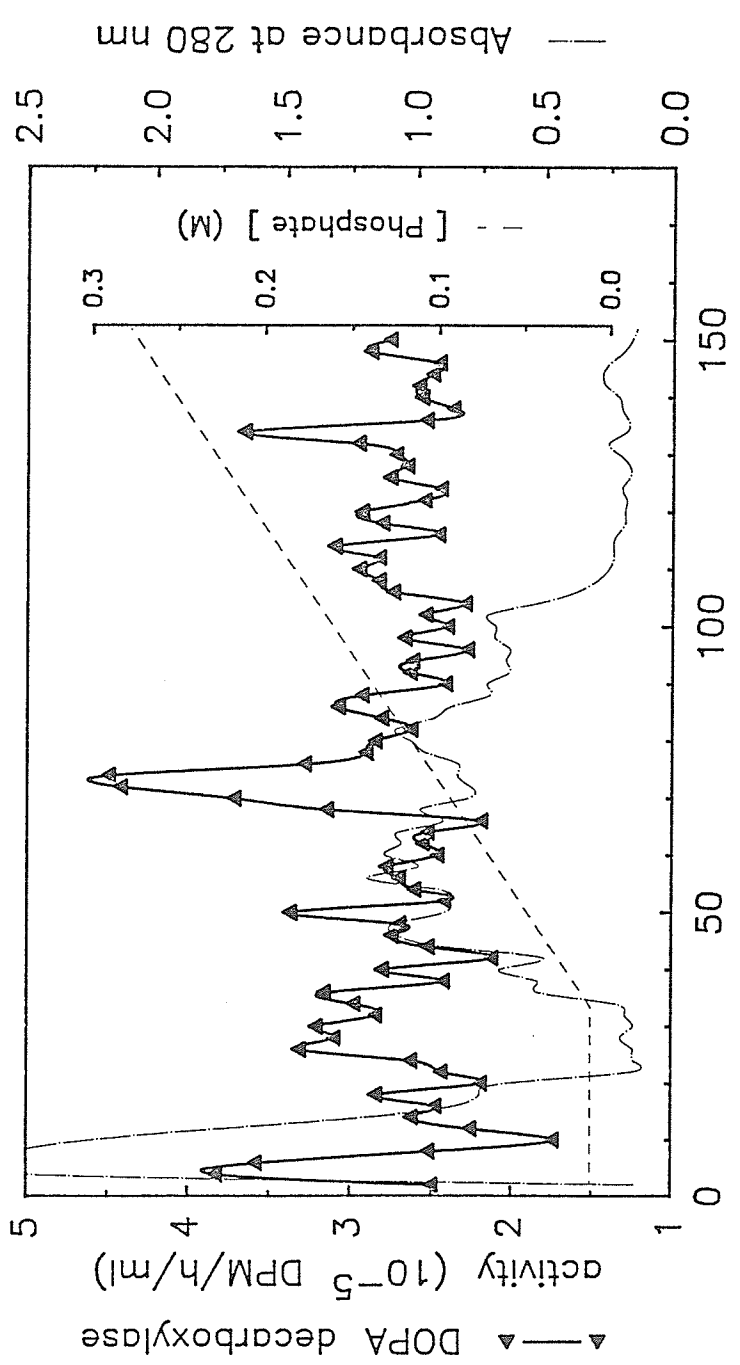

Figure 12. Chromatography of bovine striatal DOPA decarboxylase on DEAE-Sephacel. The dialyzed 30-60% ammonium sulfate fraction of the crude extract was applied to this ion-exchange column. Elution was carried out using a phosphate gradient. Enzyme activity was determined using DOPA as substrate and expressed as dopamine formed (DPM) per ml. Experimental details are described in Chapter III Section II.B.6.c.

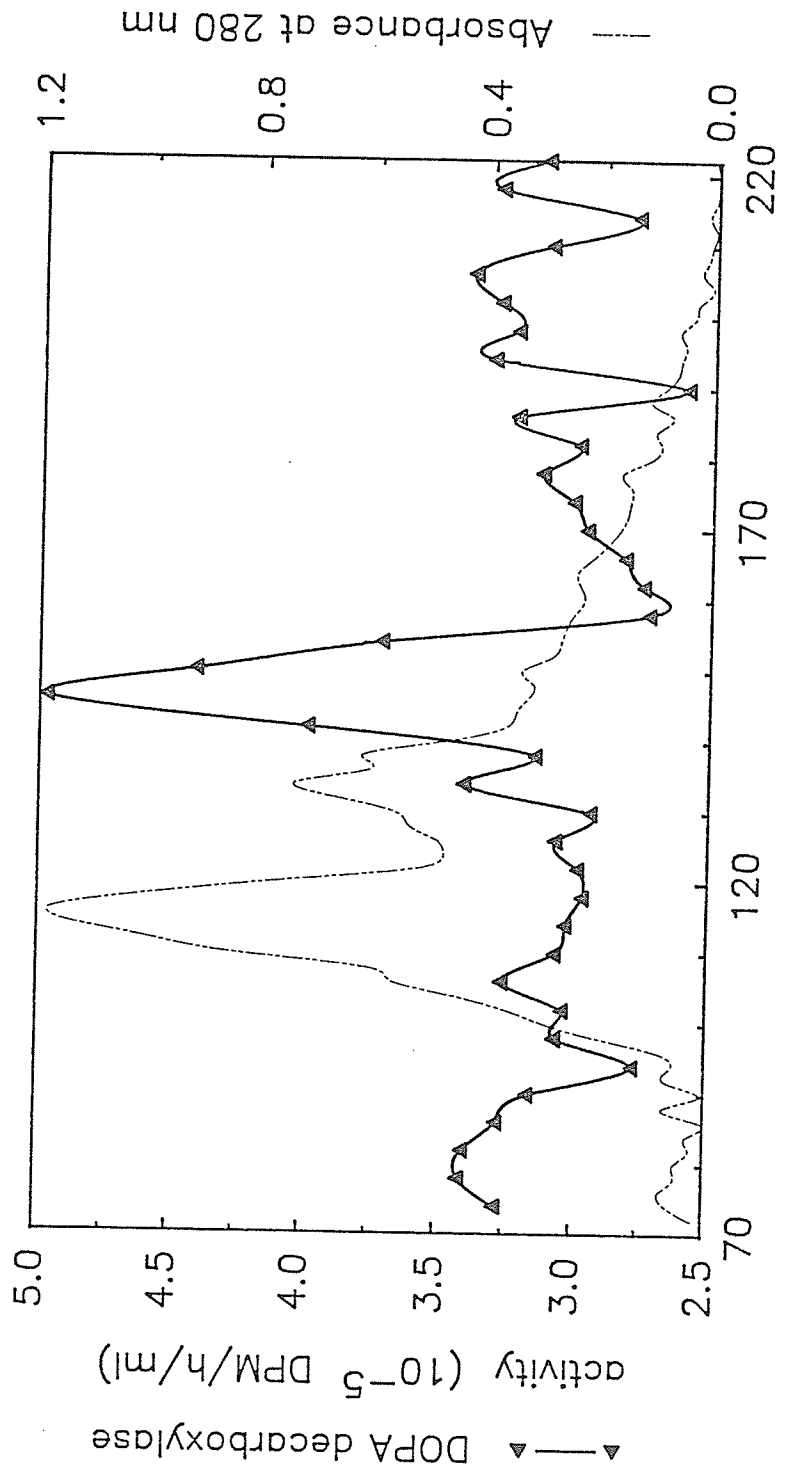

Figure 13. Chromatography of bovine striatal DOPA decarboxylase on Sephacryl S-200. The active fractions from DEAE-Sephacel column was applied to this gel filtration column. Enzyme activity was determined using DOPA as substrate and expressed as dopamine formed (DPM) per ml. Experimental details are described in Chapter III Section II.B.6.d.

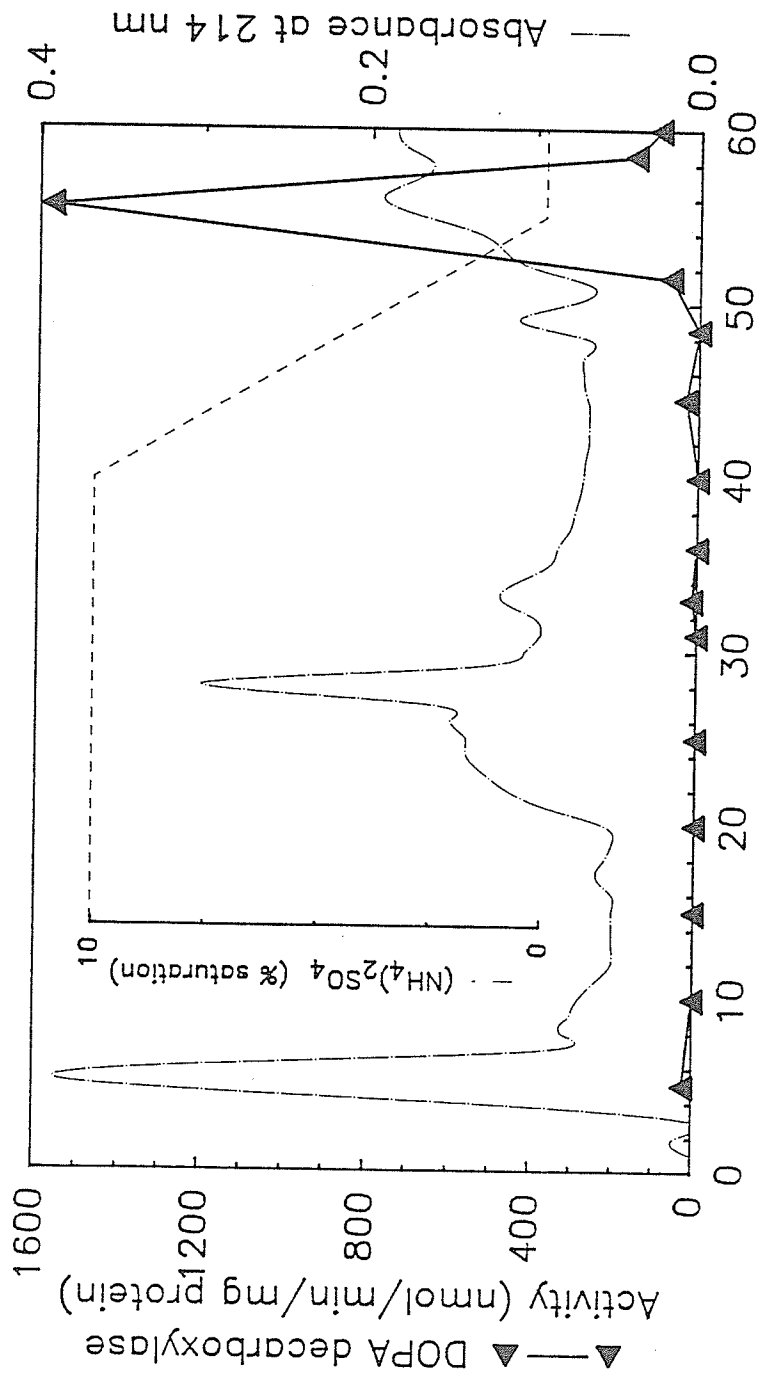

Figure 14. HPLC of bovine striatal DOPA decarboxylase on TSK Phenyl 5PW. The active fractions from Sephacryl S-200 column was injected into this hydrophobic interaction HPLC column. Elution was carried out using an ammonium sulfate gradient. Enzyme activity was determined using DOPA as substrate and expressed as nmol/min/mg protein. Experimental details are described in Chapter III Section II.B.6.e.

TABLE 23

Purification of DOPA decarboxylase from Bovine Striatum

Step	Total Protein *mg*	Total Activity *units*[a]	Specific Activity (DOPA) *units*[a]/*mg protein*	Purification *-fold*	Yield %	5-HTP decarboxylase activity *units*[b]/ *mg protein*	Ratio[c]
Crude Extract[d]	4002	4,963	1.25	1	100	0.14	8.9
Ammonium sulfate precipitation (30-60%)	1328	3,757	2.83	2.3	76	0.13	21.8
DEAE-Sephacel	58	2,293	39.7	32	46	1.61	24.7
Sephacryl S-200	3.2	613	195	156	12	10.3	18.9
Phenyl 5PW	0.19	296	1,569	1,255	6	77.6	20.2

[a]One unit of enzyme activity is defined as the amount which catalyzes the formation of 1 nmole of dopamine per min at 37°C from DOPA.

[b]One unit of enzyme activity is defined as the amount which catalyzes the formation of 1 nmole of serotonin per min at 37°C from 5-HTP.

[c]Ratio = DOPA decarboxylase activity/5-HTP decarboxylase activity

[d]Crude extract is low speed supernatant obtained from a homogenate of 10 striata (290-340 g)

observed on SDS-PAGE (Figure 15, Lane 2). Non-denaturing PAGE also shows a single broad band (Figure 16, Lane 5) suggesting heterogeneous charge distribution. This has also been observed for the DOPA decarboxylase purified from the bovine adrenals (Albert *et al.*, 1987) and rat liver (Dominici *et al.*, 1987). Using gel filtration, the relative molecular weight of bovine striatal DOPA decarboxylase was estimated to 101,000 $\pm$ 4,000 (Figure 17). We further corroborate this by sedimentation analysis of the enzyme in 5 to 20% sucrose density gradients (Martin and Ames, 1961). By comparison with several proteins of known sedimentation coefficient, the sedimentation coefficient (Davis, 1964) of DOPA decarboxylase was estimated to be 6.4 (Figure 18). The M_r of DOPA decarboxylase was calculated to be 112,000. In summary, our best preparations of bovine striatal DOPA decarboxylase showed a single band on polyacrylamide gel electrophoresis (PAGE) indicating homogeneity. The results of SDS-PAGE, gel filtration, and sedimentation analysis indicate that DOPA decarboxylase is a dimer with subunit of M_r = 56,000.

The effects of various reducing agents and sulfhydryl reagents on DOPA decarboxylase and 5-HTP decarboxylase activities were studied. Similar to the enzyme from the pig kidney (Christenson *et al.*, 1970; Dominici *et al.*, 1984b), the bovine striatal DOPA decarboxylase also requires free sulfhydryl groups for activity. Table 24 shows that DOPA decarboxylase activity was enhanced by glutathione, DTT and 2-mercaptoethanol, and was inhibited by *p*-chloromercuribenzoate and N-ethylmaleimide.

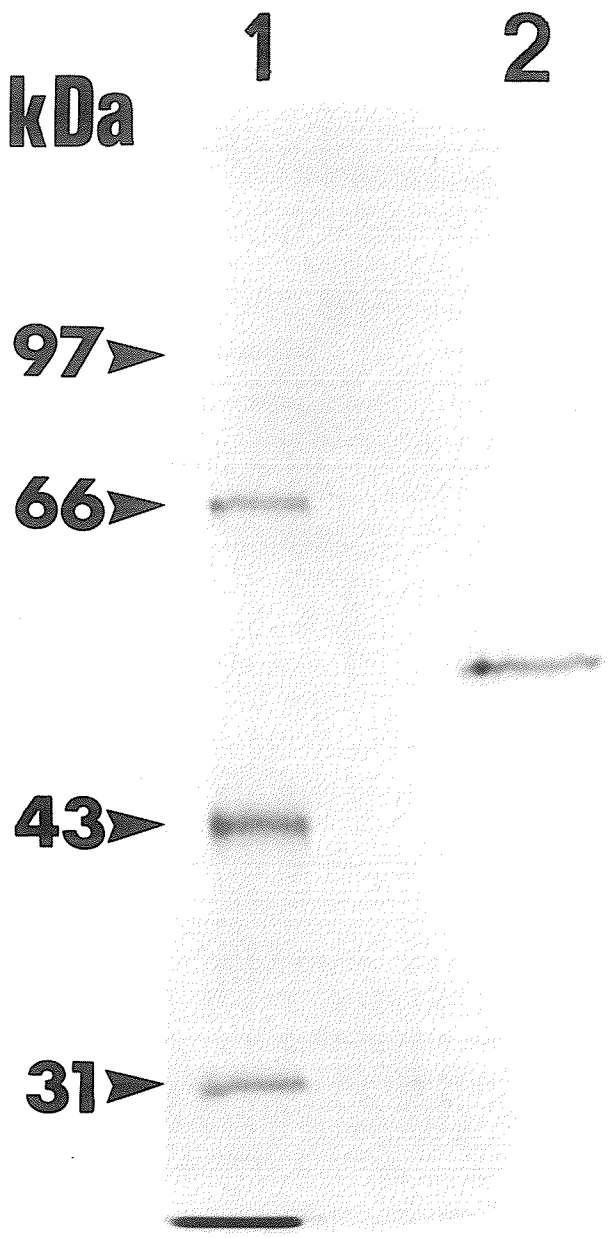

Figure 15. **SDS-PAGE analysis of bovine striatal DOPA decarboxylase.** *Lane 1*, protein molecular mass markers from *top* to *bottom* are: phosphorylase b (97,400); bovine serum albumin, (66,200); ovalbumin (43,000), and carbonic anhydrase (31,000). *Lane 2*, purified DOPA decarboxylase from the Phenyl 5PW protein. Other experimental details are as described in Chapter III Section II.B.5.

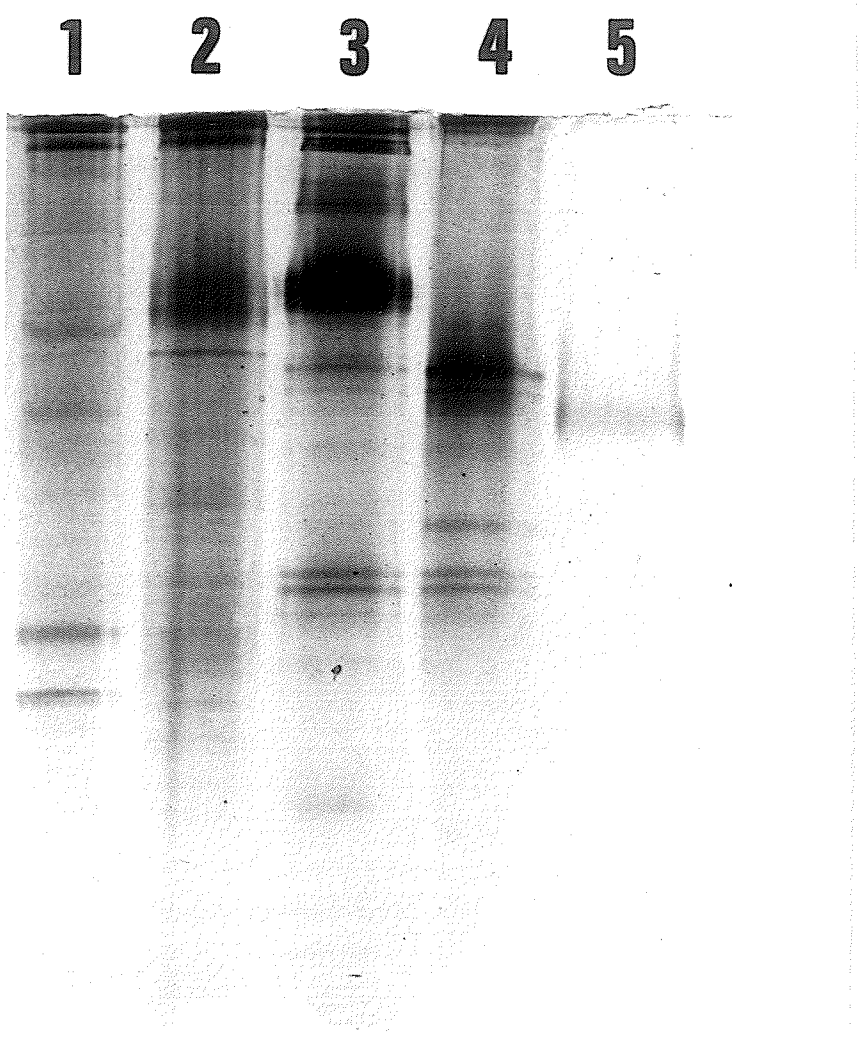

Figure 16. Discontinuous PAGE analysis of the purification sequence of bovine striatal DOPA decarboxylase. Acrylamide gels (7.5% non-denaturing) were run at pH 8.8 as described in Chapter III Section II.B.5. *Lane 1*, crude extract; *lane 2*, 30-60% ammonium sulfate fraction; *lane 3*, DEAE-Sephacel fraction; *lane 4*, Sephacryl S-200 fraction; and *lane 5*, Phenyl 5PW fraction.

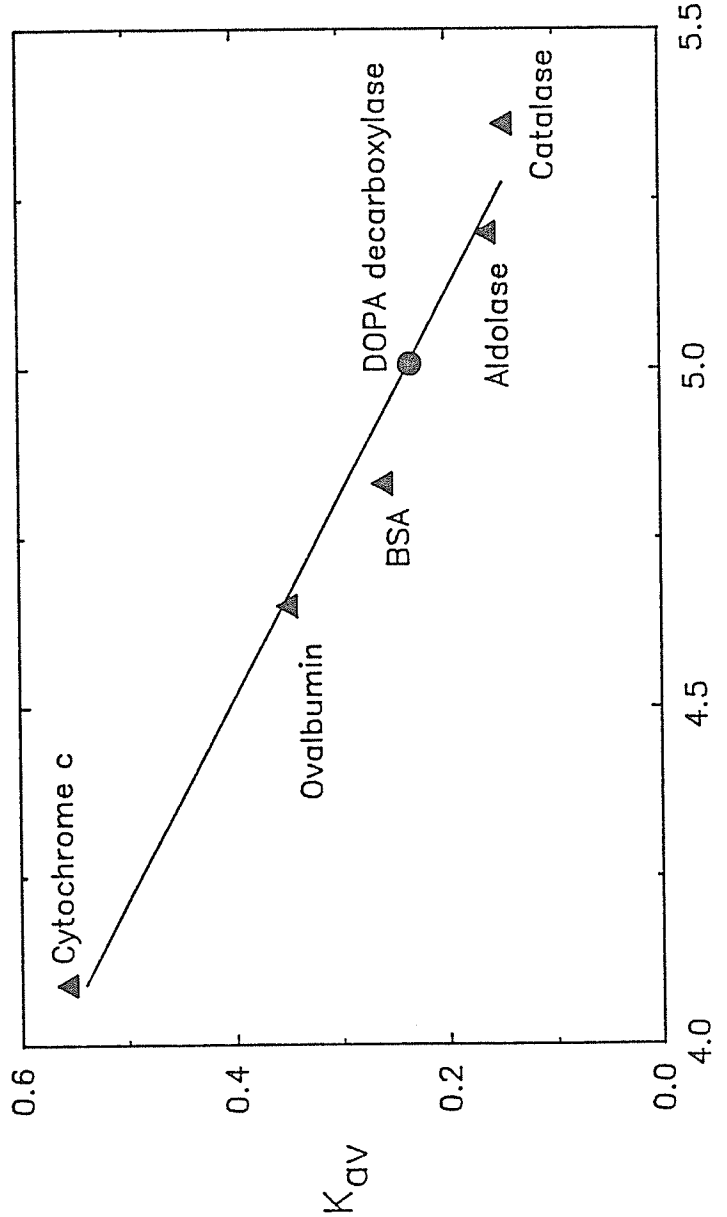

Figure 17. Estimation of the molecular weight of bovine striatal DOPA decarboxylase by gel filtration on Sephacryl S-200. Experimental conditions were as described in VII.B.6.d and the molecular weight of the decarboxylase was determined according to D'Aniello and Strazzullo (1984). Protein standards are bovine liver catalase, M_r = 232,000; rabbit muscle aldolase, M_r = 158,000; bovine serum albumin (BSA), M_r = 67,000; ovalbumin, M_r = 45,000; and horse heart cytochrome *c*, M_r = 14,000. $K_{av} = V_e-V_o/V_t-V_o$, where V_e = elution volume for the protein, V_o = column void volume (elution volume for blue dextran 2000), and V_t = total bed volume.

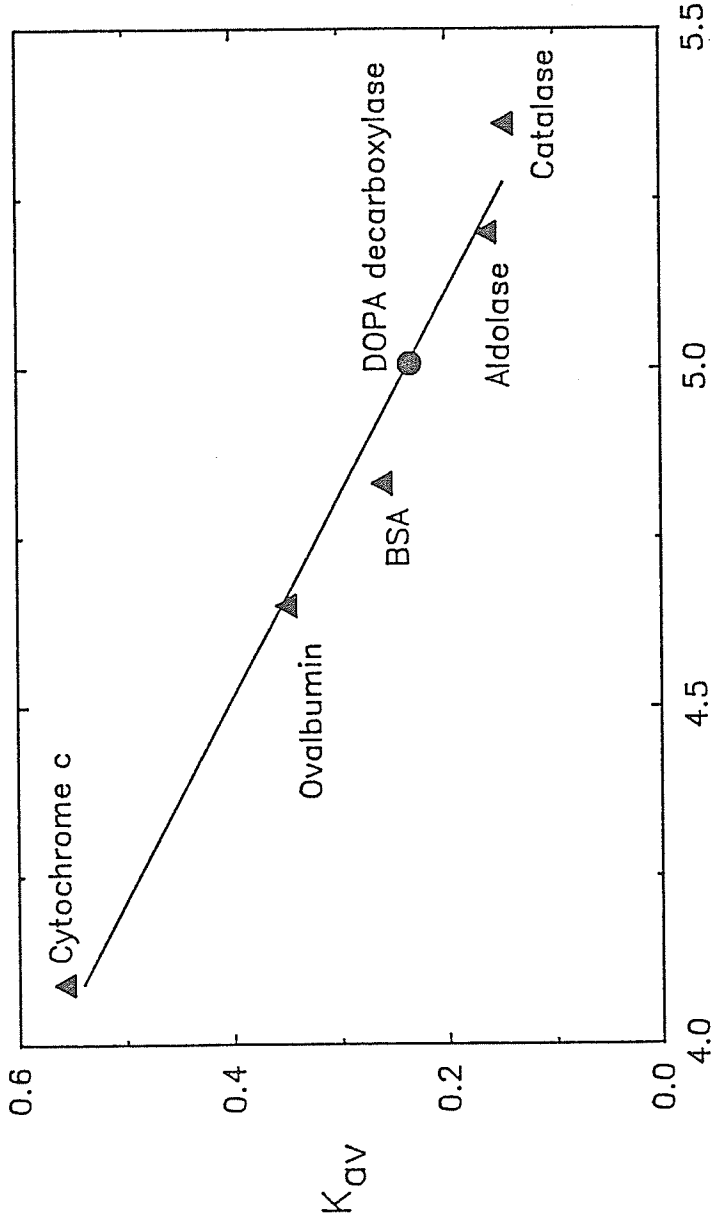

Figure 17. Estimation of the molecular weight of bovine striatal DOPA decarboxylase by gel filtration on Sephacryl S-200. Experimental conditions were as described in VII.B.6.d and the molecular weight of the decarboxylase was determined according to D'Aniello and Strazzullo (1984). Protein standards are bovine liver catalase, M_r = 232,000; rabbit muscle aldolase, M_r = 158,000; bovine serum albumin (BSA), M_r = 67,000; ovalbumin, M_r = 45,000; and horse heart cytochrome *c*, M_r = 14,000. $K_{av} = V_e - V_o / V_t - V_o$, where V_e = elution volume for the protein, V_o = column void volume (elution volume for blue dextran 2000), and V_t = total bed volume.

TABLE 24

Effects of Reducing agents and Sulfhydryl reagents on Bovine Striatal DOPA decarboxylase activity[a]

Compounds	Concentration (mM)	Enzyme Activity (%)
Control	-	100
2-Mercaptoethanol	10	154
Dithiothreitol	1	150
Glutathione	1	121
Cysteine	1	92
p-Chloromercuribenzoate	0.1	0
N-Ethylmaleimide	0.1	0

[a]A sample of the enzyme preparation was dialyzed overnight free of 2-mercaptoethanol. Aliquots of the dialyzed enzyme were then preincubated in the presence of the compounds shown in the Table for 5 min at 37°C in the assay medium prior to initiation of enzyme reaction.

The effects of certain metal ions were also investigated. Christenson and co-workers (1970) have reported that Cu^{2+}, Hg^{2+} and Zn^{2+} at 1 mM were strong inhibitors of pig kidney DOPA decarboxylase while Perry *et al.* (1969) found that at concentrations of 0.1 mM, only Hg^{2+} and Cd^{2+} inhibited guinea pig kidney DOPA decarboxylase significantly; Cu^{2+} and Zn^{2+} were only inhibitory at 1 mM. In the present study, strong inhibition of bovine striatal DOPA decarboxylase by Cd^{2+}, Cu^{2+}, Hg^{2+}, and Zn^{2+} was observed even at a concentration of 0.05 mM (Table 25). This inhibition caused by heavy metals was further examined to see whether it can be reversed by either 2-mercaptoethanol or EDTA. Table 24 shows that activity of DOPA decarboxylase could be restored completely if after inactivation with Cd^{2+} and Cu^{2+} the enzyme was incubated with EDTA, while similar treatment with 2-mercaptoethanol can only partially restored the enzyme activity. In contrast, the inactivation caused by Hg^{2+} and Zn^{2+} could be reversed only partially by 2-mercaptoethanol but not by EDTA.

Heat treatment at 50°-55°C has been used in the purification procedures of AADC from other tissues (Christenson et al., 1970; Murali and Radhakrishnan, 1970; Lancaster and Sourkes, 1972). We have now examined the effect of incubating an enzyme preparation at 55°C in an assay medium with no substrates (DOPA or 5-HTP) or coenzymes (PLP). After incubation at 55°C for 5 min, DOPA decarboxylase activity was reduced by more than 40% whereas 5-HTP decarboxylase activity was reduced by only 10-20% (Figure 19).

TABLE 25

Effects of Metal ions on Bovine Striatal DOPA decarboxylase activity[a]

Metal Ions	Enzyme Activity (%)		
	None	2-mercaptoethanol[b] (1 mM)	EDTA[b] (0.5 mM)
Control	100	135	135
Cd^{2+} (0.05 mM)	0	41	157
Cu^{2+} (0.05 mM)	13	88	128
Hg^{2+} (0.05 mM)	0	14	0
Zn^{2+} (0.05 mM)	0	82	0

[a]A sample of the enzyme preparation was dialyzed overnight free of 2-mercaptoethanol. Aliquots of the dialyzed enzyme were then preincubated in the presence of the compounds shown in the Table for 2 min at 37°C in the assay medium prior to initiation of enzyme reaction.

[b]To determine the reversibility of the effects of the metal ions, enzyme solutions were preincubated as described above, followed by the addition of 2-mercaptoethanol or EDTA. This mixture was further incubated for 3 min prior to initiation of enzyme reaction.

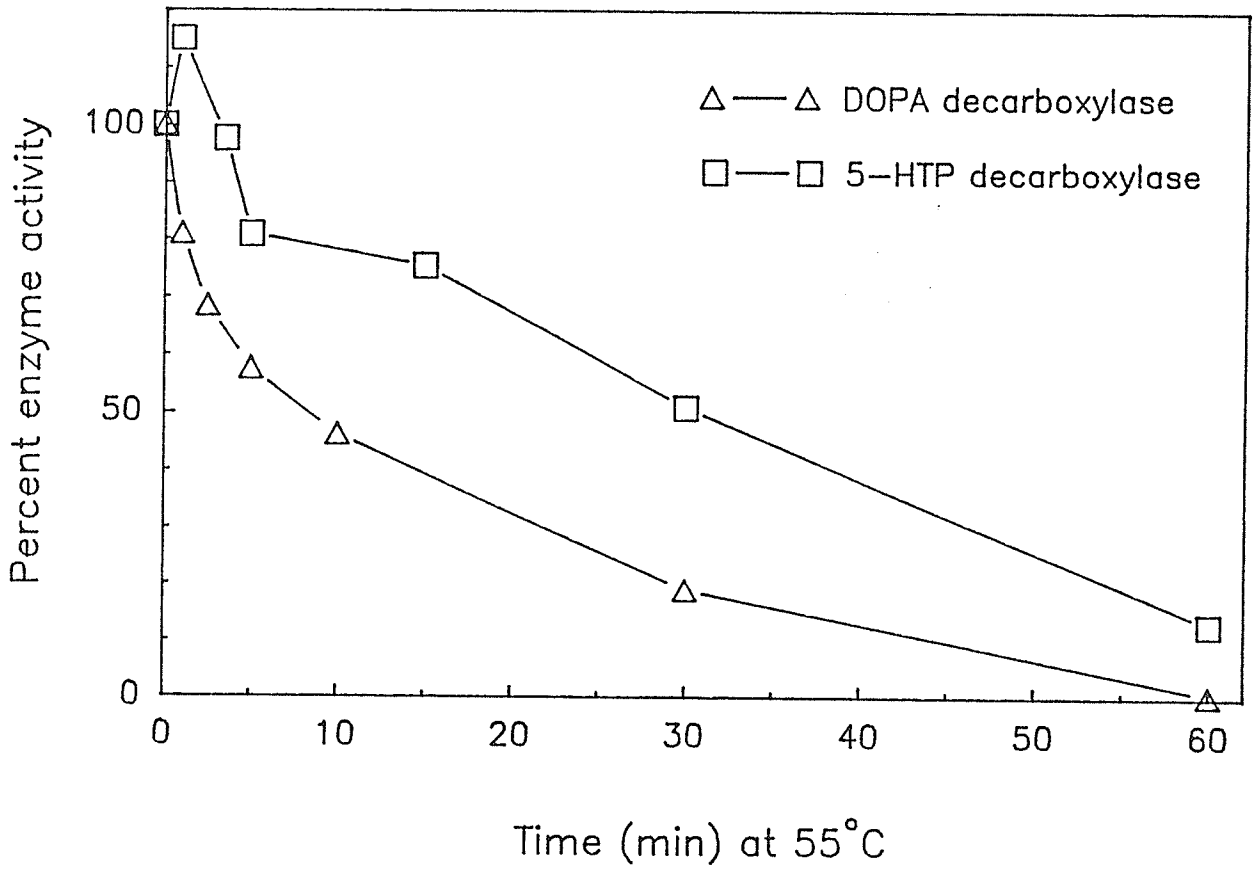

Figure 19. Heat treatment of bovine striatal DOPA decarboxylase at 55°C. Aliquots of an enzyme preparations were incubated at 55°C in the assay medium containing the buffer and 2-mercaptoethanol, for the various time indicated. Enzyme activities using either DOPA or 5-HTP as substrates were determined as described in Chapter III Section II (Part A).B.5 and .B.6, respectively.

D. Discussion

DOPA decarboxylase has now been purified to homogeneity from bovine corpus striatum to a specific activity of about 1,600 units/mg protein, which is in the same order of magnitude as those reported for the pure enzyme from non-neuronal sources. Discontinuous non-denaturing PAGE analysis of the isolated protein gave a single band indicating homogeneity. The bovine striatal DOPA decarboxylase isolated in this study also catalyzes the decarboxylation of 5-HTP. This broad substrate specificity resembles the characteristic of AADC purified from non-neuronal tissues (see Chapter II.B.4.d: *Substrate specificity*). In the crude extract, the ratio of DOPA decarboxylase activity over 5-HTP decarboxylase activity was 9 whereas after ammonium sulfate precipitation, the ratio of the two decarboxylation activities was 22 (Table 23). The reason for the enrichment of DOPA decarboxylase activity is not defined but may be due to the loss in 5-HTP decarboxylase activity after the ammonium sulfate precipitation process.

The evidence obtained from PAGE and SDS-PAGE clearly indicate a single protein capable of decarboxylating DOPA and 5-HTP. However, histidine decarboxylase was originally thought to be decarboxylated by aromatic L-amino acid decarboxylase (Lovenberg *et al.*, 1962) until the isolation of a specific and distinct histidine decarboxylase (Schwartz *et al.*, 1970). As seen in the rat brain, the distribution of the two enzyme activities also varies between several bovine brain regions. This indicates the presence of different amounts of decarboxylating activities specific for each substrate in different brain regions.

Moreover, heat treatment of the enzyme preparation inactivated the two decarboxylation activities at different rates. The results of the present study suggests that there could be a similar situation with regard to DOPA and 5-HTP decarboxylases.

The average M_r of bovine striatal DOPA decarboxylase calculated from gel elution method and sedimentation analysis is 106,000. This compares well with the molecular weight of DOPA decarboxylase purified from other tissues, which ranged from 100,000 to 112,000 (Table 26). The sedimentation coefficient of striatal DOPA decarboxylase was also the same as that reported for the pig kidney DOPA decarboxylase (Borri Voltattorni *et al.*, 1979). Based on the data obtained from SDS-PAGE analysis, we conclude that bovine striatal DOPA decarboxylase is a dimer.

Cd^{2+}, Cu^{2+}, Hg^{2+}, and Zn^{2+} all show strong affinity for ligands such as phosphates, cysteinyl, and histidyl side chains of proteins (Stokinger, 1978; Vallee and Ulmer, 1972). Hg^{2+} and Cd^{2+} also interacts with carboxyl groups, tryptophan, and tyrosine (Vallee and Ulmer, 1972). However, these two heavy metal ions are normally thought to exert their effects through their interaction with the free sulfhydryl and disulfide groups of proteins (Stokinger, 1978; Vallee and Ulmer, 1972). Hg^{2+} inhibition of the DOPA decarboxylase activity is probably through the binding of a cysteinyl group since 2-mercaptoethanol can reverse the inhibition while EDTA cannot; Hg^{2+} binds more tightly to cysteine (log K = 45.4) than to EDTA (log K = 22.1) (Sillen and Martell, 1971). Cd^{2+} may interact somewhat differently since its inhibition of enzyme

TABLE 26

Comparison of the Molecular Weights of DOPA decarboxylase from Various Sources

Tissue Source	M_r	Reference
Bovine Striatum	106,000	This study
Bovine Adrenal	112,000	Albert *et al.* (1987)
Bovine Brainstem	100,000	Nishigaki *et al.* (1988)
Human Pheochromocytoma	100,000	Maneckjee and Baylin (1983)
	100,000	Ichinose *et al.* (1985)
Rat Liver	100,000	Ando-Yamamoto *et al.* (1987)
	100,000	Dominici *et al.* (1987)
Rat Kidney	100,000-108,000	Shirota and Fujisawa (1988)
Pig Kidney	112,000	Christenson *et al.* (1970)
	103,000	Borri Voltattorni *et al.* (1979)

activity can be fully restored by EDTA and partially restored by 2-mercaptoethanol. This indicates that both Cd^{2+} and Cu^{2+} very likely also interact with side chain groups (e.g. histidyl group) other than the free sulfhydryl group. Zn^{2+} also may interact between a free sulfhydryl group and a side chain group thus causing certain conformational damage to the enzyme structure.

Aromatic L-amino acid decarboxylase (AADC), has been suggested to lack substrate specificity and to be responsible for the synthesis of both dopamine and serotonin (Lovenberg *et al.*, 1962). The immunological identity of the brain enzyme with a similar decarboxylase from the pig kidney has also been reported (Christenson *et al.*, 1972). However, much more antibody per unit of enzyme activity was required for the complete inhibition of decarboxylase activity in rat brain compared to that of pig kidney. Recently, it has been reported that a single gene codes for AADC in both neuronal and non-neuronal tissues (Albert *et al.*, 1987). However, the nearly 800 untranslated bases of the 2.3-kb AADC mRNA may yet play a role in defining the specificity of the enzyme in different tissues. The neuronal DOPA decarboxylase purified in the present study may be similar to that from non-neuronal tissues since they share many characteristics such as optimal pH, substrate specificity, molecular weight, sedimentation coefficient, sulfhydryl group requirement, and inhibition by heavy metal ions. It should be noted, however, that the neuronal DOPA decarboxylase was more heat labile than the non-neuronal enzyme. Furthermore, aminooxyacetic acid which has no effect on the adrenal enzyme either *in vitro* or *in vivo* ((Giorgi and Rubio, 1981) can inhibit the neuronal DOPA decarboxylase (see Table 21). Additionally,

α-methyl DOPA has been reported to inhibit the pig kidney DOPA decarboxylase by 71% and 98% at 0.1 and 0.5 mM concentrations, respectively (Sourkes, 1954). At similar concentrations, α-methyl DOPA inhibited only 17% and 46% of neuronal DOPA decarboxylase activity, respectively (see Table 20). The presence of more than one decarboxylase for aromatic amino acids in mammalian tissues has been suggested by various lines of evidence. Earlier work from previous sections has shown that pyridoxine deficiency has differential effects on the activity of AADC using either DOPA or 5-HTP as the substrate. Rahman *et al.* (1982) have also reported that acute effects of PLP deficiency differentially affected the recovery pattern of AADC activities toward DOPA and 5-HTP, after *in vitro* addition of PLP. Differences in the distribution of AADC activity towards DOPA and 5-HTP in different tissues and brain regions have also been shown by several investigators (Sims et al., 1973; Rahman et al., 1981; Rahman and Nagatsu, 1982). Furthermore, we have shown that the neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and its metabolite, MPP^+, enhance 5-HTP decarboxylase but not DOPA decarboxylase activity.

CHAPTER IV

GENERAL DISCUSSION

General Discussion

Aromatic L-amino acid decarboxylase is a PLP-dependent enzyme directly involved in the synthesis of the neurotransmitters, dopamine and serotonin. Throughout the last twenty years, the question whether this enzyme exists as a single protein or multiple proteins has been debated by various research groups. Many reports on neuronal AADC have also been extrapolated, directly and indirectly, from that of non-neuronal enzymes. For example, antibodies produced against the adrenal or kidney AADC have been used in immunoprecipitation and immuno-localization of brain AADC. In view of this, the report describing the immunochemical cross-reactivity between DOPA decarboxylase and histidine decarboxylase is of major significance. Especially due to the fact that prior to the identification of a specific histidine decarboxylase in different regions of the brain, it was always assumed that AADC was also responsible for the decarboxylation of histidine. This may also hold true for DOPA and 5-HTP decarboxylases. Recently, using antibodies produced against the adrenal AADC (DOPA as substrate), a cDNA for AADC has been isolated and subsequent Southern blot analysis are consistent with the presence of a single gene coding for AADC in all tissues. It is quite possible that different isoenzymic forms of AADC may arise from a single gene. These different forms may subserve specific functions in different brain regions. The laboratories of F.E. Bloom, T. Nagatsu, and A.G. Roberge have reported that the optimal conditions in terms of pH, substrate concentrations, cofactor requirements, and some kinetic characteristics are different for the decarboxylation of DOPA and 5-HTP

in the rat brain and other tissues. They suggest that this might be due to the presence of several protein species or isoenzymes of AADC.

The results of the present study lend further support to the hypothesis that the decarboxylation of DOPA and 5-HTP are regulated independently. A summary of the differences between DOPA and 5-HTP decarboxylases is presented in Table 27. Although pyridoxine deficiency decreased both DOPA and 5-HTP decarboxylase activities, the percentage decrease in activity was shown to be non-identical (Table 15). This can account for the difference in monoamine levels seen in the pyridoxine-deficient rats compared to the pyridoxine-supplemented group. Characterization of the two enzyme activities has revealed differences in the optimal conditions for the two decarboxylations and in the distribution of the enzyme activities in different regions of the rat and bovine brain. In the rat, DOPA decarboxylase from the corpus striatum also exhibited lower K_m and higher V_{max} than that from the brainstem. Dialysis of the brain homogenates, in the presence and absence of hydroxylamine, resulted in a total or greater loss of 5-HTP decarboxylase activity compared to that of DOPA decarboxylase activity, indicating that PLP is more tightly bound to DOPA decarboxylase apoenzyme than to 5-HTP decarboxylase apoenzyme. These results suggest that the synthesis of serotonin could also be limited by cofactor availability. Furthermore, the present study also shows that the neurotoxin, MPTP and its oxidation product, MPP^+, can enhance 5-HTP decarboxylase activity but not DOPA decarboxylase activity. Dissimilar responses of DOPA decarboxylase and 5-HTP decarboxylase to α-methyl DOPA, aminooxyacetic acid, hydralazine, and phenelzine have been

TABLE 27

Properties and Optimal Experimental Conditions for Brain DOPA and 5-HTP Decarboxylase

	DOPA decarboxylase	5-HTP decarboxylase
1. pH optimum	6.7	8.3
2. K_m for substrate (DOPA or 5-HTP) (μM)	165	270
3. V_{max} (pmol/min/mg protein)	1473	99.7
4. K_m for PLP (μM)	9.44	234
5. Temperature sensitivity at 55°C	50% loss after 10 min	50% loss after 30 min
6. Effect of pyridoxine deficiency	Less sensitive	Very sensitive
7. Effect of MPTP/MPP^+	Slight decrease	2-fold increase in activity
8. Inhibition by:		
α-Methyl DOPA (0.5 mM)	46%	83%
Aminooxyacetic acid (1 mM)	78%	20%
Hydralazine (0.5 mM)	78%	59%
Phenelzine (0.1 mM)	47%	22%

demonstrated as well. It is interesting to note that several studies including the present one have indicated that aminooxyacetic acid inhibits AADC in the brain while another study has shown that this carbonyl-trapping agent has no effect on the enzyme from the adrenals.

Thus far, the results are consistent with the suggestion that the decarboxylation of DOPA and 5-HTP are regulated independently. At this juncture, there is no direct evidence for the involvement of separate proteins for the decarboxylation of DOPA and 5-HTP. If separate proteins were involved in the two decarboxylations, conclusive evidence for the distinct identity of these protein entities could only come from the purification and characterization of the suggested proteins. DOPA decarboxylase has now been purified from the bovine corpus striatum and has some properties similar to the non-neuronal enzyme. A summary of the similarities and differences between the neuronal and non-neuronal enzyme is given in Table 28. The notion that this enzyme has a single active site can be discounted as heat treatment of the enzyme preparation inactivated the two decarboxylation activities in a dissimilar fashion. Similar findings have been observed in rat brain homogenates by Sims and colleagues. Although it is possible that this enzyme has two separate active sites for DOPA and 5-HTP, it should be pointed out that pyridoxine deficiency did not cause a parallel decrease in the total enzyme protein available for the two decarboxylations in different rat brain regions when assayed under optimal cofactor and substrate conditions. This non-parallel decrease was not due to a lower affinity of 5-HTP decarboxylase for PLP alone. Another possibility is that DOPA decarboxylase and 5-HTP decarboxylase may have overlapping

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6. Effect of pyridoxine deficiency	Less sensitive	Very sensitive
7. Effect of $MPTP/MPP^+$	Slight decrease	2-fold increase in activity
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Hydralazine (0.5 mM)	78%	59%
Phenelzine (0.1 mM)	47%	22%

substrate specificities similar to that of glutamic acid decarboxylase and cysteic acid/cysteine sulfinic acid decarboxylase. If this were the case, DOPA decarboxylase can decarboxylate both DOPA and 5-HTP. However, the presence of a decarboxylase specific for 5-HTP cannot be ruled out. The use of 5-HTP or DOPA interchangeably as substrates for the determination of DOPA decarboxylase or 5-HTP decarboxylase activities should be avoided as it may well provide misleading information about the anatomically and functionally different neurotransmitter systems. Dopaminergic systems has been associated with the initiation and execution of extrapyramidal movement, schizophrenia, prolactin release, growth hormone secretion, ovulation, and melanocyte-stimulating hormone release. A disturbance in serotonin metabolism has been implicated in sleep, appetite and thermoregulation, sexual behavior, pain perception and control of pituitary secretions as well as in the pathophysiology of migraine, myoclonus, and depressive illness. Moreover, this laboratory has demonstrated that a decrease in serotonin levels has led to hypertension, hypothyroidism, impairment of pineal function, and sleep disturbances. A better comprehension of the nature and properties of the enzymes responsible for DOPA and 5-HTP decarboxylations will enable us to study the regulation of these enzymes. Then the goal of restoring the brain and body's proper chemical equilibrium may eventually be achieved through development of better diagnostic and therapeutic methodologies for the various pathophysiological conditions described above.

The final resolution of the question whether one or multiple proteins are involved in the decarboxylation of aromatic amino acids in

mammalian tissues will come from the complete purification and sequence analysis of both decarboxylases involved, in neuronal and non-neuronal tissues. We now have a handle on DOPA decarboxylase but must await the purification of 5-HTP decarboxylase.

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