MECHANISMS OF LEVODOPA ACTION IN THE CENTRAL NERVOUS SYSTEM

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ABSTRACT

MECHANISMS OF LEVODOPA ACTION IN THE CENTRAL NERVOUS SYSTEM

By

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L-Dihydroxyphenylalanine (L-Dopa) is presently the most efficacious agent available for the treatment of Parkinson's disease. The antiparkinsonian action of this compound has been attributed to its ability to replenish the diminished concentrations of dopamine in the basal ganglia of patients with this disease. The objective of this study was to determine if the actions of L-dopa are totally dependent upon the conversion to dopamine, or if L-dopa per se has an independent action. This was tested by comparing the actions of L-dopa to those of D-dopa, which is not metabolized to dopamine in the central nervous system. Analysis of the behavioral actions of D- and L-dopa were conducted in a simple model in which direct and indirect dopaminergic agonists can be differentiated. One week after the unilateral intrastriatal injection of 6-hydroxydopamine, which selectively destroys dopaminergic nerve terminals on the injected side, mice circle spontaneously toward the side of the lesion (ipsilateral circling). Indirect dopaminergic agonists increased the rate of ipsilateral circling. Administration of a

direct-acting dopaminergic agonist (e.g. apomorphine) causes the mice to reverse the direction of circling towards the nonlesioned side (contralateral circling).

Intraperitoneal administration of both L- and D-dopa elicited contralateral circling, but the L-isomer was approximately ten times more potent than that of D-isomer. Optical rotation analysis revealed that samples of D-dopa were not contaminated with L-dopa. Following the administration of doses of L- and D-dopa which produced approximately equal numbers of contralateral turns per two minutes (10 and 100 mg/kg, respectively), the cerebral concentration of both isomers of dopa was approximately the same but the accumulation of dopamine occurred only after the administration of L-dopa. This experiment suggested that dopa may have direct dopaminergic receptor activating properties. The results of subsequent studies with decarboxylase inhibitors revealed that this proposal was incorrect.

Hydrazinomethyldopa, which blocks only peripheral aromatic-L-amino acid decarboxylase (AAAD) prolonged and enhanced the actions of both L- and D-dopa. Ro44602, an inhibitor of both peripheral and central AAAD activity, initially blocked and then enhanced the contralateral circling in response to both isomers of dopa. NSD 1015, which also inhibits both central and peripheral AAAD, completely blocked contralateral circling in response to both L- and D-dopa, but not to apomorphine.

In order to determine if a correlation exists between periods of active contralateral circling and increase cerebral conversion

of ^{3}H-dopa to ^{3}H-dopamine, intravenous ^{3}H-L-dopa was injected at selected time intervals following the administration of various AAAD inhibitors. During periods of increased L-dopa-induced contralateral circling in hydrazinomethyldopa or Ro44602 pretreated mice, there were concurrent increases in the concentrations of ^{3}H-dopamine in the brain. When contralateral circling to dopa was blocked, following pretreatment with Ro44602 or NSD 1015, the ^{3}H-dopamine concentrations were significantly less than control. These results indicate that the administration of the centrally-active AAAD inhibitors effectively block in vivo conversion of dopa to dopamine and there is a correlation between the number of contralateral turns/2 minutes and the increase in brain dopamine. The ability of the AAAD inhibitors to block contralateral circling induced by both D- and L-dopa suggest that these compounds must be converted to dopamine before the dopaminergic receptors are activated. It has been reported that D-Dopa can be converted to L-dopa in the kidney. It is suggested that in the presence of an AAAD inhibitor L-dopa synthesized in the kidney from D-dopa is reabsorbed and transported to the brain where small quantities are converted to dopamine, which in turn activate the supersensitive dopaminergic receptors. It cannot be excluded that some undefined biochemical action of D-dopa plays some role in the D-dopa-induced contralateral circling.

Systemic administration of L-dopa depletes the brain content of 5-hydroxytryptamine (5-HT). The abilities of L- and D-dopa to deplete forebrain 5-HT were compared to ascertain whether dopamine formation was essential for the depletion of 5-HT. Administration

of L-dopa increased forebrain dopa and dopamine concentrations with a simultaneous reduction in the concentration of 5-HT. A ten fold higher dose of D-dopa resulted in smaller increases in dopa and dopamine concentrations than observed with L-dopa. The 5-HT concentration was significantly reduced only by the highest dose of D-dopa. The depletion of forebrain 5-HT was better correlated with the increase in the dopamine concentration than with accumulation of cerebral dopa.

L-Dopa-induced reduction of forebrain 5-HT was studied in mice with unilateral lesions of the dopaminergic nerve terminals. 6-Hydroxydopamine injection into the striatum markedly reduced the dopamine content without altering the 5-HT or dopa concentrations. In the nonlesioned hemiforebrain, L-dopa administration caused a dose-related increase in the concentration of dopa and dopamine with a concomitant reduction in the content of 5-HT. The only significant difference between control and lesioned hemiforebrains was a reduction of the dopamine accumulation on the lesioned side. These results support the contention that the depletion of 5-HT occurs independent of dopaminergic nerve terminals.

MECHANISMS OF LEVODOPA ACTION IN THE CENTRAL NERVOUS SYSTEM

By

David Buell Goodale

A DISSERTATION

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to my parents, for their inspirational faith, love, and guidance

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INTRODUCTION

Over the last decade L-3,4-dihydroxyphenylalanine (L-dopa) has achieved prominence as the drug of choice in treating patients with Parkinson's disease (Hornykiewicz, 1974, 1975). The first clinical demonstration of the therapeutic efficacy of L-dopa was made independently by two groups of investigators, Birkmayer et al. (1961) and Barbeau et al. (1961). The clinical efficacy of this amino acid was attributed to its ability to be decarboxylated to dopamine in the brain, thereby replenishing the diminished levels of this amine within the basal ganglia.

At present most patients treated with L-dopa experience side effects and therefore this drug may not be viewed as an ideal therapeutic agent. The purpose of this dissertation was to examine in detail various alternative mechanisms through which L-dopa might exert its pharmacological actions within the central nervous system. The major question which this dissertation has attempted to answer is whether or not L-dopa must be decarboxylated to dopamine in order to produce its neurochemical and behavioral effects.

The application of L-dopa to the treatment of parkinsonism is an excellent example of a therapeutic advance occurring as the direct result of basic research providing insight into neuropharmacological mechanisms. A presentation of the historical development

of L-dopa as an effective antiparkinsonian agent will serve as a proper antecedent to a discussion on alternative mechanisms of action.

HISTORICAL BACKGROUND

James Parkinson (1817) in one of the classic papers of medical history, first recognized that certain neurological features were common to one disorder which he called shaking palsy or paralysis agitans. He described the disease as characterized by:

> ". . . involuntary tremulous motion with lessened musular power, in parts not in action and even when supported; with a propensity to bend the trunk forward, and to pass from a walking to a running pace; the senses and intellects being uninjured."

Since tremor need not be present and true paralysis is not a consistent manifestation of this disease, Charcot in the middle of the nineteenth century proposed that paralysis agitans was a misnomer and that the disorder should be known as Parkinson's disease. Throughout this dissertation the term parkinsonism will be used in the broad sense to encompass a clinical syndrome composed of four features: tremor, rigidity, akinesia, and loss of postural reflexes.

After Parkinson's original description of the disorder, one century elapsed before a correlation was made between the neurological deficits and specific pathological changes within the brain. Trétiakoff (1919) studied the brain of nine patients with parkinsonism. He found that there was a degeneration and reduction in the number of pigmented cells in the substantia nigra, together with cytoplasmic inclusions in some of the cells. Recently Bernheimer et

al. (1973) have confirmed and extended the original findings of Trétiakoff by comparing the neuropathological changes in the three major types of parkinsonism: postencephalitic, idiopathic (degenerative), and arteriosclerotic (senile). No major qualitative differences in the morphological changes within the substantia nigra were found among these three types of parkinsonism.

Following the suggestion by Trétiakoff that the substantia nigra was abnormal in parkinsonism, it was not until the mid-1950s that the functional significance of dopamine was appreciated. Prior to this time it had been considered simply a precursor to norepinephrine. Carlsson (1957) suggested that the central effects of reserpine might be explained on the basis of dopamine depletion since the precursor DL-dopa dramatically antagonized the reserpine-induced behavioral depression. Subsequently, Carlsson (1958) developed a fluorometric procedure for assaying dopamine which was much more sensitive and specific than previous procedures. Using this method he found the brain content of dopamine and norepinephrine to be almost equivalent. The following year the regional distribution of these two amines was found to be markedly different (Carlsson, 1959; Bertler and Rosengren, 1959a,b). The striatum contained approximately 10 μg/g of dopamine and only 0.1 μg/g of norepinephrine, thus suggesting that the function of dopamine was not merely that of a precursor.

In 1960 Ehringer and Hornykiewicz studied the brain content of dopamine and norepinephrine in patients with various neurological disorders. They found that in patients with Parkinson's disease

there was a marked reduction in the usually high levels of dopamine in the corpus striatum. This was not only direct evidence for a physiological role played by cerebral dopamine, but also suggested that the diminished levels of dopamine were causative in the genesis of the parkinsonian syndrome.

Since it had been demonstrated that catecholamines would not effectively penetrate the blood-brain-barrier (Axelrod et al., 1959; Weil-Malherbe et al., 1961; Samorajski and Marks, 1962), the immediate metabolic precursor of dopamine, L-dopa, became the logical candidate for clinical trials.

In 1961 both Birkmayer and Hornykiewicz, and Barbeau et al. independently administered L-dopa intravenously and demonstrated a marked reduction in rigidity and akinesia with little or no effect on tremor. Cotzias et al. (1967, 1969), by gradually increasing and ultimately using large oral doses, demonstrated that L-dopa could be successfully used in the clinics as an antiparkinsonian agent.

Further improvement of the therapeutic regimen was accomplished by inhibition of the peripheral route of L-dopa metabolism. In addition to 3'-O-methylation, the decarboxylation of L-dopa to dopamine has been shown to be a major route of metabolism both in the brain and in the periphery by Bartholini et al. (1967), and Baldessarini and Chace (1972). The conversion of L-dopa to dopamine, known to be mediated by the enzyme aromatic-L-amino decarboxylase (AAAD, EC 4.1.1.26) (Blaschko, 1939; Sourkes, 1966; figure 1), was demonstrated to be selectively inhibited in the periphery by drugs which did not penetrate the blood-brain-barrier such as:

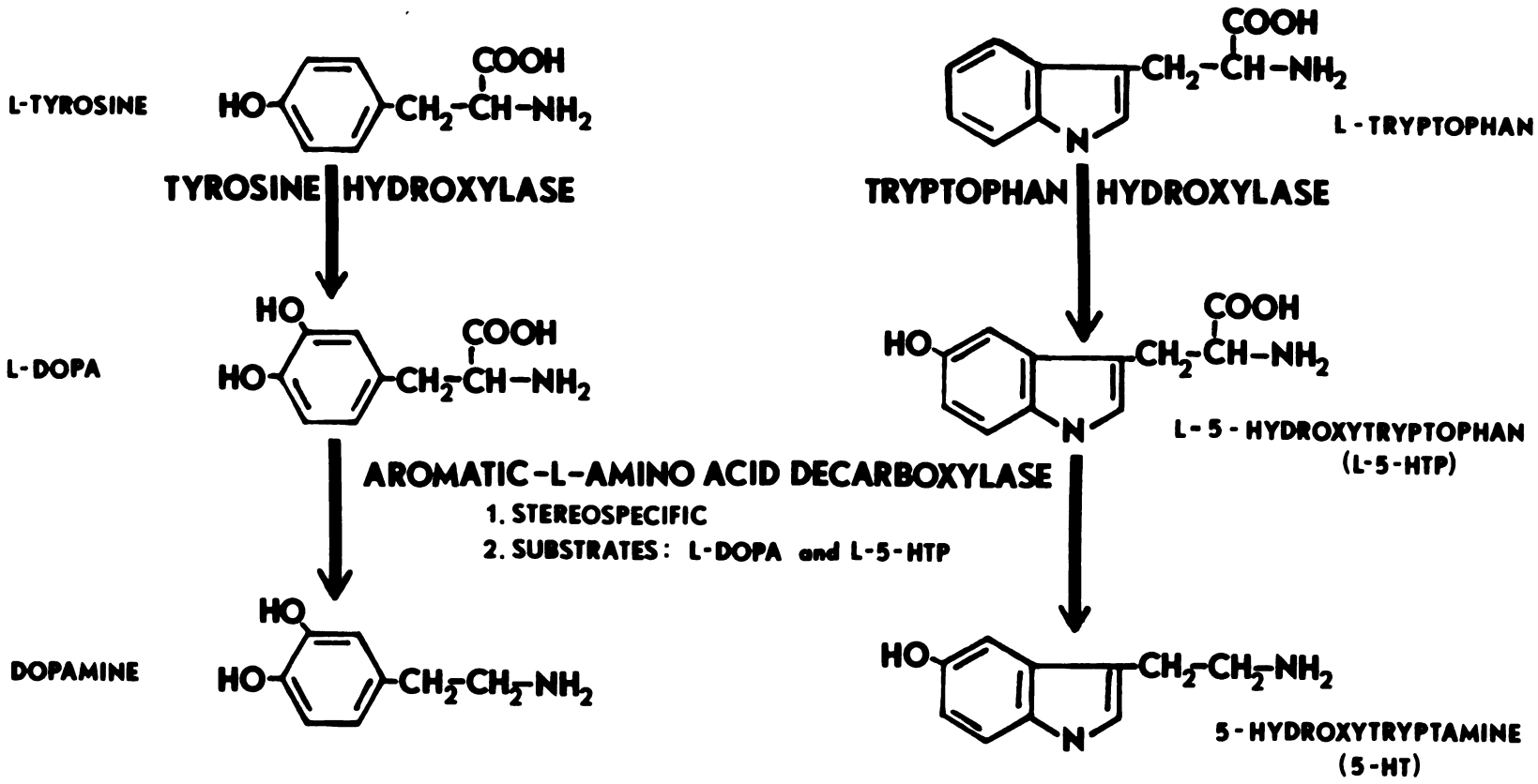

Figure 1. Pathway for the synthesis of dopamine and 5-hydroxytryptamine.

hydrazinomethyldopa (MK-486, HMD) or low doses (<100 mg/kg) of N-(DL-seryl)-N'-(2,3,4-trihydroxybenzyl) hydrazine (Ro44602) (Bartholini and Pletscher, 1969; Lotti and Porter, 1970). Experimentally, Pletscher and Bartholini (1971) found that administration of Ro44602 prior to L-dopa resulted in elevated dopa concentrations in human plasma and markedly increased rat brain dopa and dopamine contents when compared to controls receiving only L-dopa.

Subsequently the addition of Ro44602 or HMD to the L-dopa treatment schedule permitted a reduction in the dosage of L-dopa needed for a therapeutic effect (Barbeau et al., 1971 and Marsden et al., 1973) and diminished the L-dopa-induced peripheral side effects of nausea and vomiting (Calne et al., 1971; Yahr et al., 1971; Markham, 1974). Further modification of the therapeutic regimen has occurred through the availability of single dosage forms containing L-dopa combined with a decarboxylase inhibitor: Sinemet (250 mg l-dopa + 25 mg L-α-methyldopahydrazine, (Merck, Sharp & Dohme) or Madopar (200 mg L-dopa + 50 mg Ro44602, Hoffmann-La Roche) (Korten et al., 1975; Dietrickson et al., 1975; Rinne et al., 1975).

LITERATURE REVIEW

The discovery that L-dopa was therapeutically efficacious in the treatment of parkinsonism fulfilled the previous expectation that this amino acid would be decarboxylated in the brain and thereby replenish the diminished levels of dopamine within the basal ganglia. Since these initial clinical trials with L-dopa, many experiments have been reported, some supporting the prerequisite conversion of dopa to dopamine and others suggesting that dopa *per se* is mediating its behavioral and biochemical effects.

A. Dopamine as a neurotransmitter for the nigrostriatal pathway

The first evidence for a dopaminergic nigrostriatal pathway came from the application of the Falck-Hillarp fluorescence histochemical procedure which permitted the demonstration of dopamine-containing cell bodies in the pars compacta of the substantia nigra (Dahlström and Fuxe, 1964) and dopamine-containing nerve terminals in the corpus striatum (Carlsson *et al.*, 1962; Andén *et al.*, 1966; Fuxe *et al.*, 1964). Furthermore electrolytic lesions of the substantia nigra resulted in decreases in fluorescence intensity and the dopamine content of the neostriatum (Andén *et al.*, 1964; 1966b; Bedard *et al.*, 1969). Moore *et al.* (1971) applying the much more sensitive Fink-Heimer technique finally provided definitive anatomical evidence for a direct nigrostriatal dopaminergic projection. From the lesioned substantia nigra, the pathway was traced rostrally

through the medial internal capsule and lateral hypothalamic area to the corpus striatum (caudate-putamen). The complete nigrostriatal pathway was elegantly visualized by constructing a photomontage from histofluorescent parasagittal sections of an embryonic mouse brain (Golden, 1972). Finally, as a concluding study on the anatomical characterization of this dopaminergic projection, Jacobowitz and Palkovits (1974) constructed a detailed stereotaxic atlas of catecholaminergic-containing neural structures according to the coordinates of the König and Klippel (1963) rat brain atlas. The atlas constructed by Jacobowitz and Palkovits (1974) was much more detailed than earlier studies by Ungerstedt (1971a) and compared the topographical distribution of catecholamine and acetylcholinesterase-containing neurons in the rat brain.

Neuropharmacological, electrophysiological, and in vivo release studies have demonstrated that dopamine fulfills the criteria for the neurotransmitter of the nigrostriatal pathway. The enzymes necessary for the synthesis of dopamine, tyrosine hydroxylase (EC 1.14.16.2) and AAAD, have been demonstrated in the midbrain area and caudate nucleus by both immunofluorescent (Hökfelt et al., 1973a; Pickel et al., 1975a,b) and neurochemical techniques (Cicero et al., 1972; McGeer et al., 1971; Segal and Kuczenski, 1974). Furthermore, lesions of the substantia nigra result in diminished levels of these enzymes within the corpus striatum (Goldstein et al., 1969; Moore et al., 1971; Fyrö et al., 1972; and McGeer et al., 1973). A simple precursor role for dopamine is unlikely since neither the enzyme dopamine-β-hydroxylase (EC 1.14.17.1) (Hartmen and Udenfriend, 1972)

nor its product, norepinephrine (Vogt, 1954; Brock and Marsden, 1972), have been found in the caudate nucleus.

The enzymes, catechol-O-methyltransferase (COMT, EC 2.1.1.6), monoamine oxidase (MAO, EC 1.4.3.4) and aldehyde dehydrogenase (EC 1.2.1.3) are involved in the catabolism of dopamine and have been demonstrated to be present in the corpus striatum (Broch and Fonnum, 1972; Bogdanski et al., 1957; Duncan et al., 1975). The relative intra- and extraneuronal distribution of these enzymes has not been clearly differentiated since lesions causing degeneration of the nigrostriatal pathway have reported to significantly reduce (Duncan et al., 1972; Agid et al., 1973) or to have no effect (Goldstein et al., 1969; Marsden et al., 1972) on the activities of these enzymes.

The presence of enzymes for the synthesis and catabolism of dopamine within the nerve terminal region satisfies one criterion for a neurotransmitter (Cooper et al., 1974). Another criterion to be fulfilled is that dopamine must mimic the action of the transmitter released by nerve stimulation. Early microiontophoretic studies (Bloom et al., 1965; Herz and Zieglgänsberger, 1968; McLennon and York, 1967; Connor, 1970) found that dopamine inhibited extracellular neuronal activity within the corpus striatum. Subsequently, electrical stimulation within the substantia nigra also produced a predominantly depressant effect on striatal unit activity (Connor, 1968; Feltz and Mackenzie, 1969; Hull et al., 1970). Thus, dopamine applied microiontophoretically in the striatum mimics the effect of the neurotransmitter released by electrical stimulation.

When the spontaneous activity of units in the striatum was recorded after a chronic lesion of the nigrostriatal pathway (Ohye et al., 1970; Arbuthnott, 1974; Ungerstedt, 1975) an increase in the resting neuronal activity was found which is consistent with the suggestion that dopamine is an inhibitory neurotransmitter. Furthermore, when dopamine was iontophoretically applied to neurons in the denervated striatum, an enhanced sensitivity to the depressant action of dopamine was observed (Feltz and de Champlain, 1972a; Ungerstedt, 1975). The occasional excitatory responses elicited by stimulation of the substantia nigra have been attributed to a non-dopaminergic projection to the striatum (Hedreen, 1971; Feltz and de Champlain, 1972b; Hattori et al., 1973). Therefore, the above electrophysiological studies support the theory that dopamine is the inhibitory neurotransmitter of the nigrostriatal pathway.

As a third criterion for the classification of neurotransmitter, dopamine has been shown to be released from the caudate nucleus by several different methods including the push-pull cannula (McKenzie and Szerb, 1968; Riddell and Szerb, 1971), the superfusion cup technique (Besson et al., 1971) and the cerebroventricular perfusion technique (Carr and Moore, 1970). Estimation of the amount of liberated transmitter has been quantified by measurement of either the release of ^{3}H-dopamine accumulated in the striatum following intraventricular injection (Von Voigtlander and Moore, 1971a; Chiueh and Moore, 1974a) or of ^{3}H-dopamine synthesized from radio-labeled precursors (Chiueh and Moore, 1974b) or of endogenous dopamine (McLennan, 1964, 1965; Portig and Vogt, 1969; Lloyd and

Bartholini, 1975). Moreover, stimulation of the nigrostriatal pathway results in an increased release of ^{3}H-dopamine (Von Voigtlander and Moore, 1971b,c; Chiueh and Moore, 1973) while inhibition of tyrosine hydroxylase by alpha methyltyrosine (Chiueh and Moore, 1974c, 1975) or lesion of the nigrostriatal pathway (Von Voigtlander and Moore, 1973a) results in a blockade of drug-induced release of ^{3}H-dopamine.

B. L-Dopa as a dopamine replenishing agent

The foregoing studies provide experimental evidence that dopamine is the neurotransmitter for the neuronal pathway extending from the substantia nigra to the caudate nucleus. This conclusion, however, raises an important question about how L-dopa, with only a few remaining nigrostriatal neurons, compensates for the greatly deficient dopaminergic innervation of the striatum so as to be clinically efficacious in the treatment of parkinsonism. Although there is, as yet, no direct evidence that striatal dopamine repletion is the primary effect of L-dopa, several lines of evidence support this theory.

Selective destruction of dopaminergic neurons has been produced by injection of 6-hydroxydopamine near the cell bodies, axons or nerve terminals of these neurons (Ungerstedt, 1971a; Hökfelt and Ungerstedt, 1973; Constantino et al., 1973; Agid et al., 1973a). 6-Hydroxydopamine is a cytotoxic substance which is taken up and concentrated by a specific transport system of catecholaminergic neurons (Kostrezwa and Jacobowitz, 1974; Sachs and Jonsson, 1975). Once inside the neuron 6-hydroxydopamine undergoes autoxidation

which destroys the neuron by the production of reactive by-products such as hydrogen peroxide. The specificity of 6-hydroxydopamine lesions is demonstrated by the fact that serotonergic (Von Voigtlander and Moore, 1973b), cholinergic (Grewaal et al., 1974; Ladinsky, 1975) and gabergic (Jacks et al., 1972; Uretsky and Iversen, 1970) neurons remain unaffected by this treatment. Thus, intracerebral injections of 6-hydroxydopamine have been utilized as a selective method for approximating the dopaminergic neuronal degeneration in Parkinson's disease.

6-Hydroxydopamine-induced destruction of the dopaminergic nigrostriatal pathway results in an apparent supersensitivity to dopamine agonists as demonstrated by behavioral (Schoenfeld and Uretsky, 1972, 1973; Nahorski, 1975; Creese and Iversen, 1975), electrophysiological (Siggins et al., 1974) and neurochemical studies (Fibiger et al., 1974). The nigrostriatal pathway as schematically represented in figure 2 is a bilateral structure with cell bodies in the substantia nigra and nerve terminals in the corpus striatum. When this projection is unilaterally destroyed in either rats (Ungerstedt, 1971b; Corrodi et al., 1971; Mendez et al., 1975) or mice (Von Voigtlander et al., 1973b; Thornburg and Moore, 1974, 1975; Moore and Goodale, 1975) the animals spontaneously turn toward the side of the lesion since the dopamine synapse shown on the right remains functional (figure 2). Apomorphine, a direct-acting dopaminergic agonist and L-dopa reverse the direction of turning due to the lesion-induced supersensitivity of the neurons upon which dopaminergic receptors are located as shown on the left side of figure 2.

Figure 2. Relationship of postsynaptic supersensitivity to the contralateral circling induced by apomorphine or L-dopa in mice with unilateral 6-hydroxydopamine lesions of the corpus striatum.

The bilateral dopaminergic nigrostriatal pathway is schematically depicted in this coronal section of a mouse brain. The injection of 6-hydroxydopamine into the left striatum selectively destroys the dopamine nerve terminals. This unilateral degeneration of dopamine neurons results in an imbalance of the ascending nigrostriatal neuronal system since the dopamine synapse shown in the box on the right remains functional. The predominance of dopamine action in the intact side over the lesioned side causes the mouse to circle toward the side of the lesion (ipsilateral turning). During the first week after 6-hydroxydopamine the postsynaptic neurons become supersensitive to dopamine agonists. The supersensitivity has been schematically represented by an increase in the number of receptors. Drugs that interact directly with dopaminergic postsynaptic receptors should cause a predominance of dopamine action on the lesioned side because of the lesion-induced supersensitivity of the postsynaptic neurons. Apomorphine, a direct dopaminergic receptor agonist induces turning away from the lesioned side.

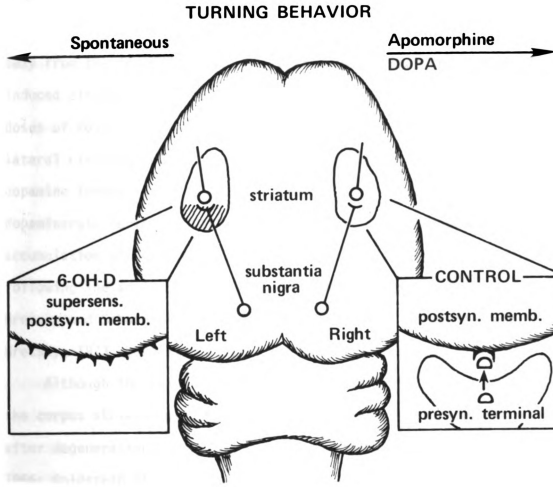

Figure 2. Relationship of postsynaptic supersentivity to the contralateral circling induced by apomorphine or L-dopa in mice with unilateral 6-hydroxydopamine lesions of the corpus striatum (modified after Moore, 1974).

In summary, rodents with a unilateral lesion of the nigrostriatal pathway may be utilized to study the mechanism of action of compounds which induce circling away from the lesioned side by activating supersensitive receptors on the lesioned side.

Ungerstedt (1971b) demonstrated that in rats with unilateral degeneration of the nigrostriatal pathway L-dopa produced circling away from the lesioned side. He further showed that the L-dopa-induced circling response was delayed by pretreatment with high doses of Ro44602 (500-1000 mg/kg). Thus, the L-dopa-induced contralateral circling response was considered to be the direct result of dopamine formed in the corpus striatum lacking a full complement of dopaminergic nerve terminals. Subsequent studies demonstrated the accumulation of dopamine in 6-hydroxydopamine-lesioned animals following the administration of L-dopa (Poirier et al., 1967; Uretsky and Schoenfeld, 1971; Lytle et al., 1972; Schoenfeld and Uretsky, 1973; Langelier et al., 1973).

Although the exact site of this decarboxylation is not known, the corpus striatum was found to contain decarboxylase activity after degeneration of the nigrostriatal pathway (Andén et al., 1966; Goldstein et al., 1969). The decarboxylation has been proposed to occur in 5-hydroxytryptamine (5-HT)-containing nerve terminals (Butcher et al., 1970; Barrett and Balch, 1971) or possibly in special catecholamine-accumulating cells that have been observed in the lesioned corpus striatum (Hökfelt and Ungerstedt, 1969; Ungerstedt, 1971b).

Nonenzymatic decarboxylation has also been proposed as a mechanism for converting L-dopa to dopamine (Sandler, 1971; Vogel, 1969; Vogel et al., 1970, 1972; LaPlante and Tran, 1972; Dairman and Christenson, 1972). However, Sims et al. (1973) and Sims and Bloom (1973) found no significant amount of decarboxylation in boiled homogenates from rat brain. Thus, nonenzymatic mechanism does not appear to be of physiological significance for cerebral decarboxylation.

Agid et al. (1973b) have proposed an additional site where L-dopa may be decarboxylated in the parkinsonian striatum. Following partial destruction of the nigrostriatal pathway, they found an increase in the specific activity (nCi/μg) of dopamine formed from ^{3}H-tyrosine in the remaining dopaminergic neurons. Thus, a hyperactivity of the dopaminergic synthetic enzymes, such as increased AAAD activity, might partially compensate for the decreased number of dopaminergic nerve terminals in the parkinsonian striatum by converting more L-dopa to dopamine. By this theory the rate of synthesis and release of dopamine formed from L-dopa is greatly increased in the remaining dopaminergic neurons, resulting in more dopamine being available to the striatal receptors. From the preceding studies it is evident that both postsynaptic (increased sensitivity of postsynaptic neurons to dopamine) and presynaptic (increase in the synthesis and release of dopamine) supersensitivity may play important compensatory roles in the response of the parkinsonian striatum to the administered L-dopa.

Two other neuropharmacological models have been used to demonstrate the necessity for decarboxylation of L-dopa. First, Bunney

et al. (1973) reported that the intraperitoneal administration of L-dopa (75-100 mg/kg) or apomorphine (0.10 mg/kg) would depress the firing rate of dopamine-containing cells in the substantia nigra zona compacta. This response was thought to occur following stimulation of striatal dopamine receptors which initiate neuronal feedback inhibition of the dopamine-containing cells in the substantia nigra. Inhibition of AAAD by Ro44602 (800 mg/kg) prevented the depression of cell firing due to L-dopa (100 mg/kg), but did not affect the apomorphine response.

The prerequisite conversion of L-dopa to dopamine has also been studied in the modulation of adenosine 3',5'-monophosphate (cyclic AMP) content in the corpus striatum (Garelis and Neff, 1974). In this system adenylate cyclase (EC 4.6.1.1) has been proposed to be the postsynaptic dopamine "receptor" (Kebabian et al., 1972; Iversen, 1975a,b; figure 3). Low concentrations of dopamine or apomorphine increased the brain content of cyclic AMP (Kebabian et al., 1972; Clement-Cormier et al., 1975; Iversen et al., 1975) while pretreatment with dopamine receptor blocking agents inhibit this response (Clement-Cormier et al., 1974; Miller, et al., 1974a).

Stimulation of the dopamine sensitive adenylate cyclase has been used to investigate the direct receptor activating properties of L-dopa. When incubated with homogenates of rat caudate nucleus L-dopa was found to have about 10% of the potency of dopamine (Sheppard and Burghardt, 1974). However, Miller et al. (1974b) found no elevation of adenylate cyclase when the incubation mixture included both L-dopa and the decarboxylase inhibitor, NSD 1055 (2.0 mM). Thus, the stimulation of adenylate cyclase by L-dopa in the

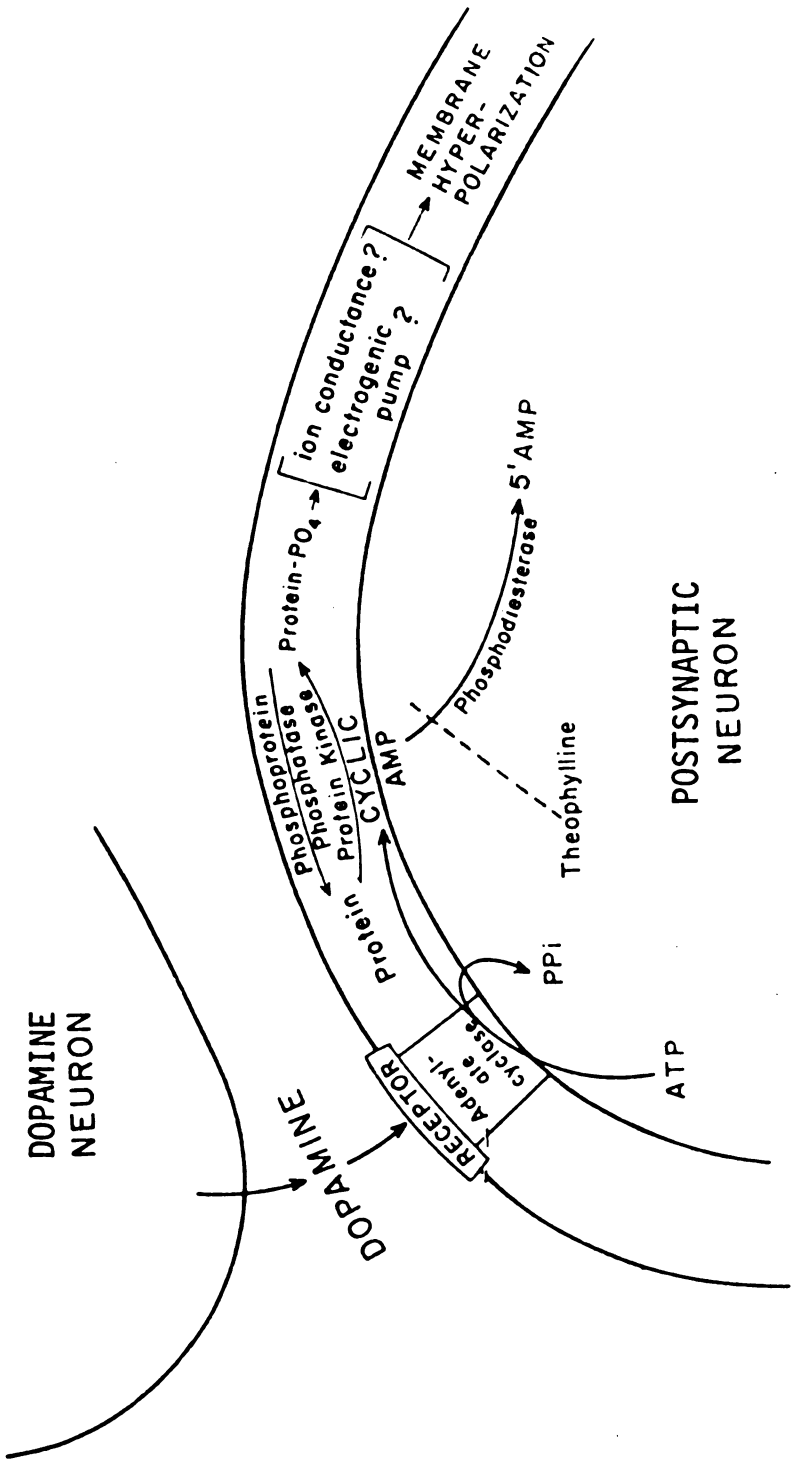

Figure 3. Schematic representation of the theoretical mechanism by which cyclic AMP mediates dopaminergic transmission (modified after Greengard et al., 1972).

previous studies (Sheppard and Burghardt, 1974) may have been the result of dopamine formed during the incubation.

The stimulation of adenylate cyclase by L-dopa has recently been demonstrated in vivo. Garelis and Neff (1974) administered L-dopa (100 mg/kg, i.p.) to rats five minutes before sacrifice and found an increase in the striatal cyclic AMP concentration. This response was blocked by pretreatment with an inhibitor of brain AAAD, Ro44602 (500 mg/kg), suggesting that L-dopa elevates striatal cyclic AMP following a prerequisite conversion to dopamine.

Experimentally Fuxe and Ungerstedt (1974) emphasized the importance of cyclic AMP in the behavioral response to L-dopa by demonstrating that inhibition of the enzyme which metabolizes cyclic AMP, phosphodiesterase (EC 3.1.4.1, figure 3), enhanced the L-dopa and apomorphine-induced circling responses in rats with unilateral nigrostriatal lesions. This interaction of L-dopa with cyclic AMP has clinical relevance since papaverine, an inhibitor of phosphodiesterase, has paradoxically been found to antagonize the therapeutic action of L-dopa in Parkinson's disease (Duvoisin, 1975).

Further support for adenylate cyclase mediating postsynaptic responses to L-dopa is that the supersensitivity which follows destruction of the nigrostriatal pathway is correlated with an enhancement of striatal adenylate cyclase response to dopamine. Indeed, Mishra et al. (1974) have reported such an effect in the rat. However, Von Voigtlander et al. (1974) did not find any

change in the sensitivity of adenylate cyclase following 6-hydroxydopamine lesions in the mouse corpus striatum. Chronic administration of chlorpromazine which also produces supersensitivity failed to alter the adenylate cyclase activity in rat striatal homogenates (Rotrosen, 1975). Therefore, although adenylate cyclase seems to play a role in the behavioral and possibly the clinical responses to L-dopa, the exact nature of that role remains to be defined.

Clinically, it has been difficult to biochemically confirm a dopamine-replenishing action for L-dopa. A positive correlation has been demonstrated between decreased numbers of cell bodies in the substantia nigra and diminished levels of dopamine in the corpus striatum of parkinsonian patients (Bernheimer et al., 1973). Although a direct correlation also exists between plasma levels of L-dopa and the therapeutic efficacy (Birkmayer et al., 1973), no therapeutic correlation exists with the dopa, dopamine, and homovanillic acid content in the cerebrospinal fluid (Papavasiliou et al., 1973; Chase, 1970). These antithetical results may be understandable if the major source of cerebrospinal fluid dopa and metabolites originates from the capillaries as found by Bartholini et al. (1971).

Lloyd and Hornykiewicz (1970a) have demonstrated that the AAAD activity although reduced by 90% of control patients was still detectable in discrete brain regions of the parkinsonian patient. Furthermore, samples taken at autopsy (Davidson et al., 1971; Rinne et al., 1974) or from biopsy (Constantinidis et al., 1974) indicate that the metabolism of L-dopa in the parkinsonian brain was similar

to laboratory animals. Thus, patients who were being treated with chronic oral L-dopa until death had 10-15 fold higher levels of dopamine and its metabolite, homovanillic acid, in the caudate nucleus than in patients which had not been treated with L-dopa. A correlation has also been reported between the clinical improvement following L-dopa therapy and the increase in brain dopamine (Lloyd et al., 1973).

C. Evidence that L-dopa acts independently of dopamine formation

The literature contains relatively few studies supporting the possibility that dopa per se is mediating its behavioral and biochemical effects. In 1973 Howse and Matthews reported that a peripheral and central AAAD inhibitor (brocresine) potentiated rather than inhibited the L-dopa therapeutic response of parkinsonian patients. Other investigators using α-methyl-dopa, which enters the brain and inhibits AAAD (Murphy and Sourkes, 1961), also demonstrated an enhanced therapeutic effect of L-dopa in Parkinson's disease (Fermaglich and O'Doherty, 1971a,b; Sweet et al., 1972). One explanation for these unexpected findings is that the AAAD is not completely inhibited in the brain, hence dopamine formation still occurs.

Behavioral studies by Smith (1962) and Vazquez and Sabelli (1975) have supported a direct action of L-dopa. Both of these authors pretreated mice with the AAAD inhibitor, α-methyl-dopa, and found a potentiation of the locomotor stimulation produced by L-dopa at high doses. Vazquez and Sabelli further supported a direct

action of dopa by demonstrating that α-methyl-dopa (500 mg/kg) inhibited the AAAD activity to 95% of control. Recently, the depressant effects of L- (178 to 320 mg/kg) and D-dopa (320 to 1000 mg/kg) on Sidman avoidance were compared and reported in abstract form (Thut, 1974). He demonstrated that inhibition of peripheral AAAD enhanced the depressant effect of L-dopa, but not that of D-dopa. Inhibition of cerebral AAAD by Ro44602 (500 mg/kg) partially reduced the depressant effect of L-dopa but did not alter the depressant effect of D-dopa. Thus, Thut suggested that only part of the depressant action of L-dopa was due to central decarboxylation.

Biochemically, an action of L-dopa independent of dopamine formation has been suggested for the depletion of 5-HT (Fahn et al., 1975). The latter investigators proposed that L-dopa reduced forebrain 5-HT content by competing with L-tryptophan for uptake into the brain. The latter results will be contrasted later with studies showing the importance of dopamine formation in the depletion of 5-HT.

An important fact which should be emphasized is that there have been no reports in the literature of the accidental or purposeful treatment with a central AAAD inhibitor which precipitated adverse reactions during L-dopa therapy. Thus, there has been no direct clinical evidence to disprove the theory that dopa per se is capable of activating dopaminergic postsynaptic receptors in Parkinson's disease.

D. L-Dopa and displacement of brain 5-hydroxytryptamine

1. Competition for aromatic-L-amino acid decarboxylase

In rodents, high doses of L-dopa (200-400 mg/kg, i.p.) significantly deplete the brain of 5-HT (Everett and Borcherding, 1968; Butcher and Engel, 1969; Narotzky, 1973) while simultaneously increasing the concentration of 5-hydroxyindoleacetic acid (5-HIAA), the major metabolite of 5-HT (Bartholini et al., 1968; Brown et al., 1975). Algeri and Cerletti (1974) further demonstrated a negative correlation between the dopa accumulated following administration of L-dopa and the forebrain 5-HT concentration. Since the AAAD enzymes which decarboxylate either L-dopa or 5-hydroxytryptophan (5-HTP) have been found to be indistinguishable both immunologically (Christenson et al., 1972; Hökfelt et al., 1973a,b) and chemically (Dairman et al., 1975), L-dopa may reduce 5-HT content simply by competing with 5-HTP for the AAAD enzyme as depicted in figure 4, step 2.

2. Displacement of 5-hydroxytryptamine from storage granules by dopamine

An alternative mechanism through which L-dopa depletes serotonin by inducing 5-HT release from neuronal storage granules has been studied by various investigators. Bartholini et al. (1968) proposed that since brain 5-HIAA concentrations were increased, then L-dopa must be displacing 5-HT from intraneuronal storage sites and thereby releasing 5-HT (figure 4, step 3). Evidence for such a

Figure 4. Diagrammatic representation of the various metabolic pathways through which L-dopa may induce different biochemical and behavioral effects.

The illustration depicts a serotonergic and dopaminergic nerve terminal in hypothetical juxtaposition. In the top half of the figure potential pathways for L-dopa inducing 5-HT depletion are presented. L-Tryptophan is converted by tryptophan hydroxylase (step 1) to L-5-hydroxytryptophan (5-HTP) which is then converted to 5-hydroxytryptamine (5-HT) by aromatic-L-amino acid decarboxylase (AAAD; step 2). High concentrations of L-dopa may compete with 5-HTP for AAAD (step 2) and a depletion of 5-HT result because of synthesis inhibition. L-Dopa may be decarboxylated and the dopamine displace 5-HT from the storage granule (step 3). L-Dopa may be decarboxylated extraneuronally (step 5) and the dopamine displace the 5-HT after uptake by the amine transport system (step 6). L-Dopa may reduce the neuronal uptake of L-tryptophan thus reducing 5-HT content by synthesis inhibition (step 4).

Various alternative pathways for L- and D-dopa activating postsynaptic dopaminergic receptors are presented in the bottom half of the figure. L-Tyrosine is converted by tyrosine hydroxylase (step 7) to L-dihydroxyphenylalanine (L-dopa) which is decarboxylated by AAAD (step 2) to dopamine. Exogenously administered L-dopa may be taken up by the neuron and decarboxylated to dopamine which is then released (step 8) and activates postsynaptic receptors.

Alternatively, L-dopa *per se* may activate postsynaptic dopaminergic receptors (step 9). D-Dopa may exert its central nervous system effects by direct dopaminergic receptor activation (step 10). D-Dopa, since it is not a substrate for aromatic-L-amino acid decarboxylase, is not converted to dopamine (step 12).

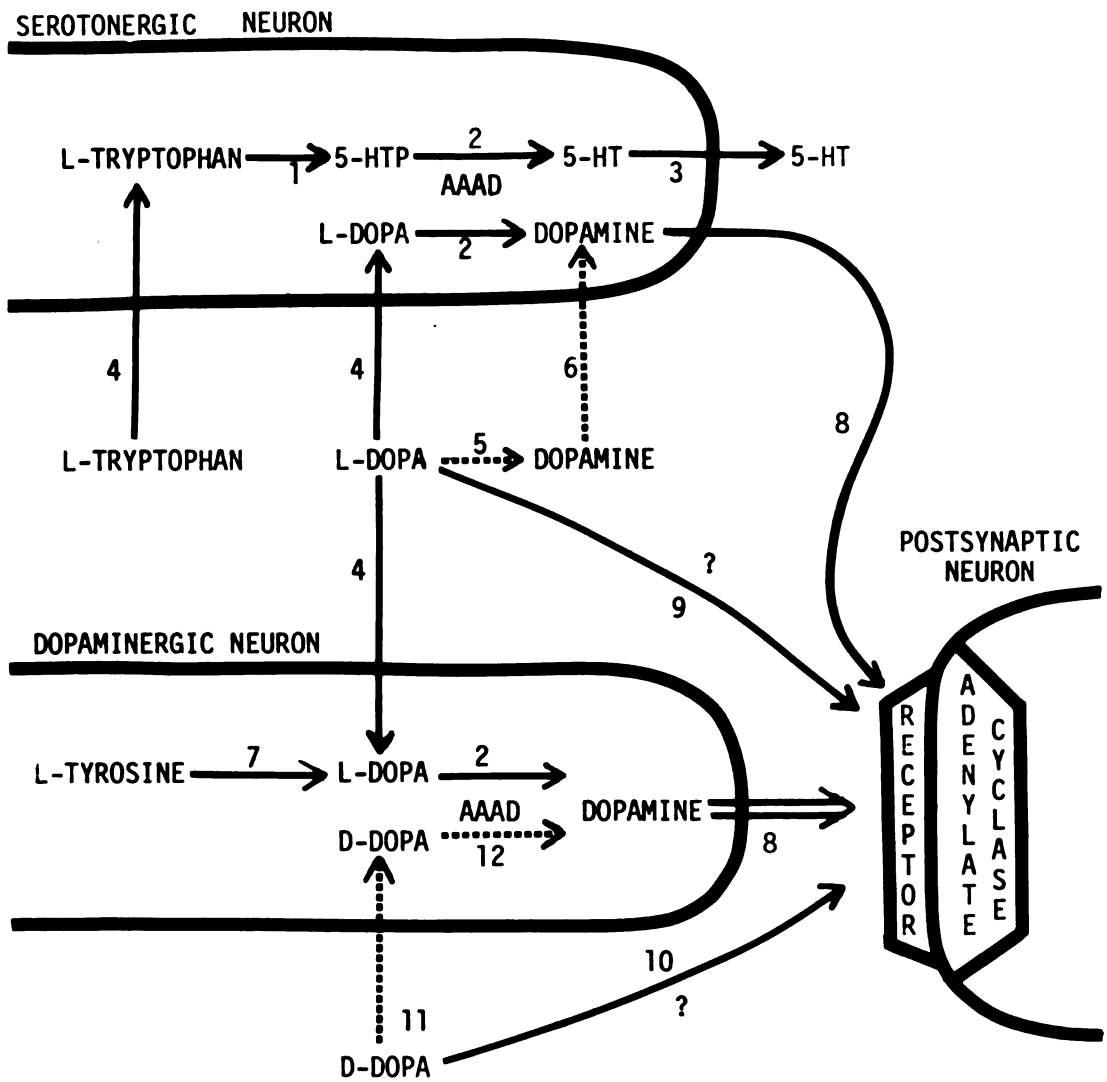

Figure 4. Diagrammatic representation of the various metabolic pathways through which L-dopa may induce different biochemical and behavioral effects.

mechanism has come primarily from *in vitro* studies where L-dopa has induced the release of ^{3}H-amines accumulated in homogenates and/or slices of rat brain (Ng *et al.*, 1970, 1971, 1972a,b; Heikkila *et al.*, 1975). Ng and coworkers further demonstrated that the releasing action of L-dopa could be blocked by MK 485 (DL-α-methyl-dihydroxy-phenylalanine hydrazine), a decarboxylase inhibitor. Thus it appears as if the conversion to dopamine (figure 4, step 2) is a prerequisite in the mechanism of action of L-dopa in releasing 5-HT *in vitro*.

Although these studies tacitly suggest that dopamine is formed inside serotonergic neurons, Friedman and Gershon (1972) suggested that the decarboxylation of L-dopa may occur before uptake into serotonergic nerve terminals (figure 4, steps 5 and 6). In support of this theory Ng *et al.* (1970, 1972b) found that dopamine ($1x10^{-5}$M) was capable of releasing ^{3}H-5-HT from rat brain slices. However, Fuller and Perry (1975) found that the 5-HT depletion by L-dopa was not blocked by an inhibitor of the amine uptake system of serotonergic neurons (figure 4, step 6) indicating that L-dopa is transported into the serotonergic neuron as the amino acid (figure 4, step 4) and is decarboxylated to dopamine which displaces 5-HT from storage vesicles (figure 4, step 3).

One physiological response thought to be mediated by 5-HT release is the L-dopa-induced hypotension (Robson, 1971; Antonaccio and Robson, 1973, 1973). Although not a common side effect, L-dopa-induced hypotension has been produced in some parkinsonian patients (Watanabe *et al.*, 1970; McDowell, 1970).

The L-dopa depletion of brain 5-HT following specific degeneration of dopaminergic nerve terminals has been studied in an effort to demonstrate the independence of dopamine formation and 5-HT depletion from dopaminergic nerve terminals. *In vitro* Ng *et al.* (1972a) found that following such lesions the formation of dopamine and the accumulation and release of ^{3}H-5-HT remained relatively unaffected. These results suggest that 5-HT is released (figure 4, step 3) by dopamine derived from the decarboxylation of L-dopa within serotonergic neurons (figure 4, step 2).

Following intraventricular 6-hydroxydopamine-induced degeneration, Uretsky and Schoenfeld (1971) and Lytle *et al.* (1972) demonstrated *in vivo* that the formation of dopamine and depletion of 5-HT by L-dopa were unaffected. In contrast, Poirier *et al.* (1971) and Langelier *et al.* (1973) have reported that electrocoagulation of the nigrostriatal pathway significantly enhances the depletion of 5-HT by L-dopa. Both of the latter studies, however, fail to report the control concentration of 5-HT on the lesioned side prior to L-dopa, leaving the question of specificity of the lesion completely unanswered.

3. Competition between L-dopa and L-tryptophan for uptake into the brain

The L-dopa depletion of forebrain 5-HT has been attributed to a competitive decrease in brain tryptophan concentration (Karobath, 1971). Since tryptophan hydroxylase *in situ* is not saturated with substrate (Costa and Meek, 1974) compounds may directly regulate

5-HT synthesis through an action on the uptake of tryptophan across the blood-brain-barrier (Fernstrom and Wurtman, 1971, 1972; Tagliamonte et al., 1971, 1973; Grahame-Smith, 1971; Biggio, 1974; Modigh, 1975; Lytle et al., 1975) or on the uptake into serotonergic neurons (Neckers and Sze, 1975). The mechanism of this inhibition may be explained by a competition between L-dopa or L-tryptophan for a common neutral amino acid transport system into the brain (Guroff and Udenfriend, 1962) or into the serotonergic neuron (figure 4, step 4). Evidence for L-dopa reducing 5-HT concentration by this mechanism comes from the report of a simultaneous decrease in endogenous tryptophan and 5-HT concentration one hour after the injection of L-dopa (Karobath, 1971). Furthermore, L-dopa was shown to inhibit the synaptosomal accumulation of exogenous ^{3}H-L-tryptophan and its subsequent conversion to ^{3}H-5-HT (Karobath, 1972).

This mechanism for the L-dopa-induced depletion of 5-HT is further supported by Butcher et al. (1972) who administered L-dopa and 5-hydroxytryptophan (5-HTP) separately and concurrently to rats after peripheral decarboxylase inhibition with low doses of Ro44602. These investigators found that 5-HTP restored 5-HT content in L-dopa treated rats thereby compensating for the decreased tryptophan concentrations. However, the latter results also support the theory of a competition between 5-HTP and L-dopa for AAAD (figure 4, step 2). Therefore, Fahn and coworkers (1975) administered tryptophan concurrently with L-dopa to determine if the decrease in forebrain 5-HT was associated with decreased tryptophan concentrations.

Indeed, tryptophan restored 5-HT concentrations to within the normal range while not altering the increased dopa and dopamine. This theory explains the decrease in 5-HT but fails to account for the simultaneous rise in 5-HIAA content seen following L-dopa (Bartholini et al., 1968). The finding by Fahn et al. (1975) of the tryptophan reversal of 5-HT depletion may have clinical relevance in the study of L-dopa therapy since L-tryptophan has been found to reverse the mental side effects of L-dopa in parkinsonian patients (Coppen, 1972; Lehman, 1973; Miller and Neiburg, 1974).

E. D-Dopa literature review

1. Transport across the blood-brain-barrier

Previously, the D-isomer of dopa has been considered not to enter the brain (Yoshida et al., 1963 and Van Rossum et al., 1969). However, studies by Oldendorf (1971) have demonstrated that the blood-brain-barrier is not completely stereospecific. Thus, D-amino acids, although transported less efficiently than the L-amino acids, do enter the brain. This stereospecificity has been demonstrated to be different for each amino acid studied (Lajtha and Toth, 1963). Furthermore, Oldendorf (1973) has recently shown that the blood-brain-barrier is more stereoselective for L-dopa than for most other amino acids studied.

Bertler et al. (1966) utilized the fluorescent histochemical technique to demonstrate that L-dopa (80 mg/kg), but not D-dopa (500 mg/kg), produced a diffuse increase in parenchymal fluorescence in the mouse brain. In addition, Shindo and coworkers (1971) using

^{14}C-D-dopa found that unlike L-dopa, which is avidly taken up and concentrated in certain regions, D-dopa has a very low uniform distribution throughout the rat brain.

2. Metabolism in the body

Early experiments indicated that exogenous D-dopa undergoes transformation in vivo, resulting in the formation of urinary dopamine (Pellerin and D'Iorio, 1955; Weiss and Rossi, 1963). Sourkes and coworkers (1964) proposed that 3,4-dihydroxyphenylpyruvate (DHPP) might represent a deaminated intermediate between D-dopa and L-dopa in the formation of urinary dopamine. Isolation of DHPP by Shindo and Maeda (1974) has confirmed that this is a major metabolite of D-dopa in the kidney (figure 5). Following the intravenous injection of ^{14}C-D-dopa, the uptake into the brain was slower and more uniform when compared to the L-isomer (Shindo et al., 1971, 1972). However, D-dopa remained in the brain a much longer time, the concentration being higher than the L-isomer at 3, 6, and 24 hours (Shindo et al., 1973a). Shindo et al. (1973b) also did not find any trace of ^{14}C-dopamine or its metabolites in the brain following the administration of ^{14}C-D-dopa. This latter finding is teleologically understandable since the enzyme which would metabolize D-dopa, D-amino acid oxidase, is not present in the mammalian forebrain (Goldstein, 1966). The finding that D-dopa is not converted to dopamine in the brain is supported by Weiss et al. (1974) who demonstrated that following administration of D-dopa

Figure 5. Major metabolic pathway of D-dopa in rat kidney.

Evidence for this metabolic pathway was established by analyzing the metabolites of ^{14}C-D-dopa *in vivo* and *in vitro* (Shindo and Maeda, 1974). After administration of ^{14}C-D-dopa to rats the urine was analyzed and found to contain dopamine as the major metabolite with a small amount of 3,4-dihydroxyphenylpyruvate (DHPP) being present. Incubation of ^{14}-D-dopa with rat kidney homogenates or slices also resulted in dopamine being the major metabolite and DHPP being the minor metabolite. No DHPP was formed following incubation of ^{14}C-L-dopa with rat kidney homogenates.

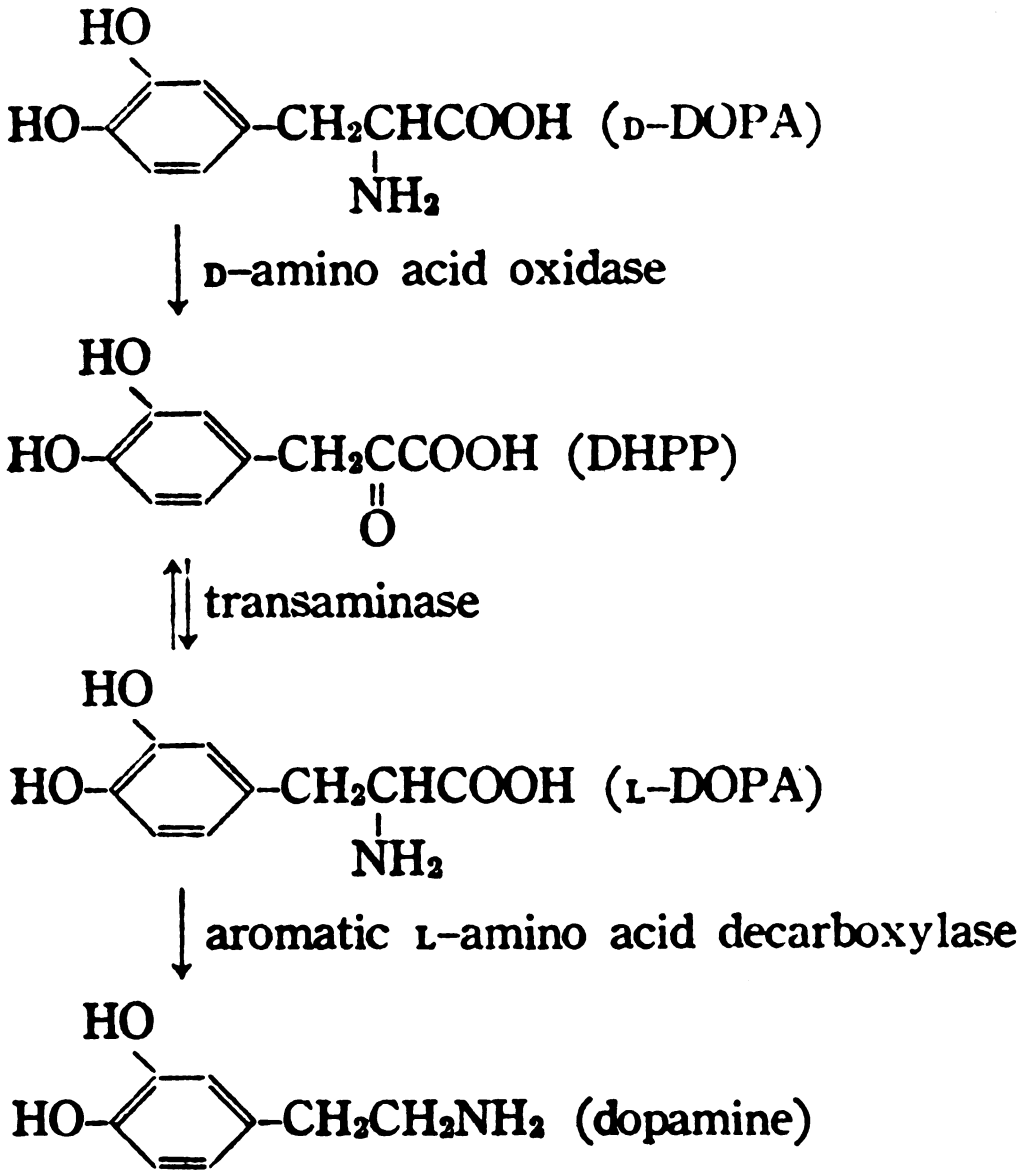

Figure 5. Major metabolic pathway of D-dopa in rat kidney (modified after Shindo and Maeda, 1974).

(500 mg/kg) no significant increase was seen in the endogenous brain dopamine content.

Therefore, studies for this dissertation originally included D-dopa for the purposes of comparison to L-dopa, since the D-isomers had previously been considered not to be converted to dopamine. Thus, any similar behavioral or biochemical actions between L- and D-dopa would be considered indicative of dopa acting directly and not through a prerequisite conversion to dopamine.

F. Aromatic-L-amino acid decarboxylase (AAAD)

1. Characterization

In 1938 Holtz and coworkers discovered an enzyme in guinea pig kidney which converted dopa to hydroxytyramine and CO_2. The enzyme was subsequently referred to as aromatic-L-amino acid decarboxylase because it catalyzed the decarboxylation of many aromatic amino acids such as: L-dopa, tyrosine, 5-HTP, tryptophan and phenylalanine (Lovenberg, 1962). The stereospecificity of this enzymatic reaction was originally determined by measuring manometrically the CO_2 produced during incubation of d(+) and d(-) dopa with the enzyme (Blascko, 1942). Using purified enzyme preparations the stereospecificity of AAAD has been confirmed by determining spectrofluorometrically (Lovenberg et al., 1962) or radiometrically (Sims et al., 1973) the amount of dopamine formed during incubation of the enzyme with D- or L-dopa. Thus, it has been established that AAAD will catalyze the conversion of L-dopa to dopamine but will not convert D-dopa to dopamine.

Originally dopa decarboxylase (EC 1.1.1.26) and 5-HTP decarboxylase (EC 1.1.1.27) were considered to be distinct enzymes (Clark et al., 1954). More recently, Christenson et al. (1972) have reported that cerebral dopa and 5-HTP decarboxylase enzymes were inhibited by antibodies against purified kidney AAAD. These authors suggested that one protein catalyzed the decarboxylation of both dopa and 5-HTP. However Sims et al. (1973) have demonstrated that dopa and 5-HTP decarboxylase activities have widely different kinetic optima for pH, temperature and substrate concentration. Furthermore, intraventricular 6-hydroxydopamine reduced dopa decarboxylase without affecting 5-HTP decarboxylase activity (Sims and Bloom, 1973). However, Dairman et al. (1975) using 6-hydroxydopamine and 5,6-dihydroxytryptamine could not demonstrate a preferential decrease in either dopa or 5-HTP decarboxylase. Therefore, the question of whether dopa decarboxylase and 5-HTP decarboxylase represent distinct enzymes has not been completely resolved.

Cerebral AAAD has been characterized as a "cytoplasmic" enzyme (Sims et al., 1973) with the highest regional activity in the corpus striatum (Lloyd and Hornykiewicz, 1970b). The endothelium of cerebral capillaries has also been demonstrated to contain a substantial amount of decarboxylase activity (Bertler et al., 1966; Constantinidis, 1968; de la Torre, 1973). This decarboxylase activity may serve as an enzymatic blood-brain-barrier converting L-dopa to dopamine which does not penetrate into the brain parenchyma (Bertler, 1966).

1. Pharmacological inhibition

Prior to the 1960s the best available inhibitors of AAAD were structural derivatives of cinnamic acid (Clark, 1959). Subsequently, the benzylhydrazines and benzoxyamines were demonstrated to be more potent inhibitors of decarboxylase activity *in vivo* (Burkard *et al.*, 1962; Hansson *et al.*, 1964). Ro44602, a benzylhydrazine, was demonstrated to inhibit the decarboxylase activity in the brain as well as in the periphery (Burkard *et al.*, 1962). Since these initial studies, the Ro44602-induced inhibition of cerebral AAAD has been more completely characterized than other AAAD inhibitors.

The inhibition of Ro44602 was first shown to be maximal in the brain 30 minutes after intraperitoneal administration (Burkard *et al.*, 1964). Subsequently, the decarboxylase activity slowly recovered and at three hours after administration was approximately 40% of control activity.

Small doses of Ro44602 (50 mg/kg) were shown to preferentially inactivate the peripheral decarboxylase activity (Bartholini, 1967). The larger doses of Ro44602 (100, 200, and 300 mg/kg) caused a dose related inhibition of decarboxylase to 75, 45 and 20% of control activity, respectively (Bartholini, 1967). The concurrent inhibition of extracerebral decarboxylase and administration of ^{14}C-L-dopa resulted in a marked elevation of ^{14}C-dopamine in the brain without elevation of catecholamines in extracerebral tissues (Bartholini and Pletscher, 1968, 1969). Thus, the utility of

Ro44602 for inhibiting AAAD in both the peripheral and central nervous system was well established in the previous studies.

In addition to inhibition of AAAD activity, Ro44602 has been demonstrated to inhibit catechol-O-methyltransferase (Burkard et al., 1964; Baldessarini and Chace, 1972). Another action of Ro44602 was shown to be a small 20% reduction of endogenous norepinephrine and 5-HT content when administered at 700 mg/kg (Pletscher and Gey, 1963).

Behaviorally, Ro44602 was demonstrated to not inhibit spontaneous locomotor activity (Pletscher et al., 1964). However, when Ro44602 was used in combination with L-dopa there was a stimulation locomotor activity at low doses of Ro44602 (50 mg/kg) Bartholini et al., 1968; Thut, 1970; Lotti and Porter, 1970). At higher doses (500 mg/kg) Ro44602 inhibited the locomotor stimulant actions of L-dopa (Bartholini et al., 1968; Lotti and Porter, 1970). Thus, Ro44602 is a potent inhibitor of cerebral AAAD and a valuable tool in studying the mechanism of action of L-dopa.

G. Turnover of 5-hydroxytryptamine in 6-hydroxydopamine-lesioned animals

An interaction, either direct or indirect, has been proposed between catecholamine and serotonergic neuronal systems (Blondaux et al., 1973). These investigators demonstrated that one week after the intracisternal administration of 6-hydroxydopamine to rats the 5-HT turnover rate was increased when compared to control rats. Peters et al. (1974) have confirmed this finding following

the administration of 6-hydroxydopamine to newborn rats. This treatment resulted in an increased rate of 5-HT synthesis when brains from the adult rats were analyzed. On the other hand, Hery et al. (1973) have reported that 6-hydroxydopamine pretreatment reduced the formation of ^{3}H-5-HT during the light period and stimulated labeled 5-HT synthesis during the dark phase. In the studies by Blondaux et al. (1973) and Hery et al. (1973) 6-hydroxydopamine was administered into the cerebrospinal fluid and this route of administration has been shown to cause a greater degeneration of noradrenergic neurons than dopaminergic neurons (Bloom et al., 1969; Bell et al., 1970). Thus, no clear differentiation was made in the previous studies for which catecholamine neurons (noradrenergic or dopaminergic) are mediating the alterations in 5-HT synthesis rates.

Interneuronal regulation of 5-HT metabolism by catecholamine neurons has been demonstrated in only one nervous system area: the pineal gland (Brownstein, 1975). The activity of serotonin N-acetyltransferase, which converts 5-HT to melatonin in the rat pineal gland, was elevated after the administration of L-dopa (Deguchi and Axelrod, 1972). Furthermore, when the noradrenergic fibers innervating the pineal gland were severed chronically, L-dopa induced much more serotonin N-acetyltransferase than in the innervated pineal gland. Although subsequent studies have determined that norepinephrine is controlling the serotonin N-acetyltransferase activity (Deguchi and Axelrod, 1973a,b), no experimental

evidence is available demonstrating that a dopaminergic neuronal system controls 5-HT synthesis rate.

The synthesis rate of 5-HT has been studied in the central nervous system as an indication of the functional status of the neuron. The turnover rate assumes that a steady state exists where the synthesis and transport of 5-HT into a metabolic pool equals its release and degradation (Tozer et al., 1966). Several methods have been utilized to estimate the turnover rate of 5-HT by measuring: the initial accumulation (Millard et al., 1972) or subsequent decline (Neff et al., 1971) of ^{14}C-5-HT following an intravenous injection of ^{14}C-tryptophan; the rate of 5-HIAA accumulation following administration of probenecid; the rate of 5-HT accumulation or 5-HIAA decline after inhibition of monoamine oxidase (Tozer et al., 1966); and most recently the accumulation of 5-HTP after decarboxylase inhibition (Carlsson et al., 1972). Experiments concerning 5-HT turnover in this dissertation have utilized the method of inhibiting the metabolism of 5-HT through monoamine oxidase and quantifying the initial accumulation of 5-HT. Turnover studies have indicated that there is no end product inhibition of 5-HT synthesis at the tryptophan hydroxylase step (Lin et al., 1969a; Millard et al., 1972. Thus, the inhibition of monoamine oxidase by pargyline administration has been demonstrated to be a reliable method for determining 5-HT synthesis rates in vivo (Morot-Gaudry, 1974).

H. Utility of 5,7-dihydroxytryptamine for producing selective depletion of brain 5-hydroxytryptamine

Recently, 5,6-dihydroxytryptamine has been introduced as a pharmacological tool for producing selective degeneration of 5-HT-containing neurons in the brains of mammals (Baumgarten et al., 1971, 1972; Costa et al., 1972; Daly et al., 1973; Victor et al., 1974). However, the reduction of forebrain 5-HT was moderate and was accompanied by limited nonspecific tissue damage and degeneration of dopaminergic neurons close to the site of injection (Baumgarten and Schlossberger, 1973; Daly et al., 1973; Saner et al., 1974). Subsequently, 5,7-dihydroxytryptamine has been studied and found to be neurotoxic to 5-HT neurons with some degeneration of noradrenergic neurons and little nonspecific damage to brain tissue (Daly et al., 1974; Baumgarten and Lachenmayer, 1972; Björklund et al., 1973; Baumgarten et al., 1973, 1975; Gershon and Baldessarini, 1974). Due to the lack of nonspecific tissue damage higher doses of 5,6-dihydroxytryptamine could be administered and therefore it was considered a potentially better drug for producing profound depletions of 5-HT. In contrast to the lack of regeneration by catecholamine neurons destroyed with 6-hydroxydopamine (Thoenen and Tranzer, 1973), 5-HT neurons regenerate following 5,6-dihydroxytryptamine (Baumgarten et al., 1974) and noradrenergic neurons regenerate following 5,7-dihydroxytryptamine (Björklund et al., 1975).

Studies were designed to investigate the specificity of 5,7-dihydroxytryptamine after unilateral injection into the corpus striatum of the mouse brain. Following demonstration of the selective degeneration of 5-HT neurons, this experimental model would be further utilized to determine the role of 5-HT neurons in the mechanism of action of L-dopa.

MATERIALS AND METHODS

A. Methods

1. Mouse turning behavioral studies

Male Swiss Webster mice (18-20 grams) obtained from Spartan Research Animal, Inc. (Haslett, Michigan) were used throughout these experiments. Groups of twelve mice were maintained in clear Plexiglass cages (45x12x13 cm). In addition to water and solid Lab Blox food (Allied Milk, Inc., Chicago, Illinois) the diets were supplemented with Sego (Very Chocolate) liquid diet food (Pet Inc., St. Louis, Missouri).

The injection of selective cytotoxic agents into the striatum of the mouse brain was performed as described previously by Von Voigtlander and Moore (1973b). Following induction of anesthesia with methoxyflurane, the head of a mouse was placed in a plastic mold (see figure 6). Four microliters of redistilled water containing 0.8 micrograms (μg) sodium ascorbate and 10, 25, 50, or 100 μg 5,7-dihydroxytryptamine creatinine sulfate or 16 μg 6-hydroxydopamine were injected slowly over a 60-90 second time period. Since an inverse correlation has been demonstrated between apormophine-induced contralateral circling and depletion of striatal dopamine (Thornburg and Moore, 1975), the extent of dopamine depletion following 6-hydroxydopamine lesions was estimated seven days later by the subcutaneous injection of apomorphine (0.25

Figure 6. Sagittal view of the apparatus for making injections into the corpus striatum of the mouse brain.

The plastic head mold indicated by the cross-hatched area surrounds and immobilizes the head of an anesthetized mouse. The anterior and lateral coordinates of the injections are regulated by the length of the nylon cuff on the needle of the syringe. The grey area represents the corpus striatum of the mouse telencephalon.

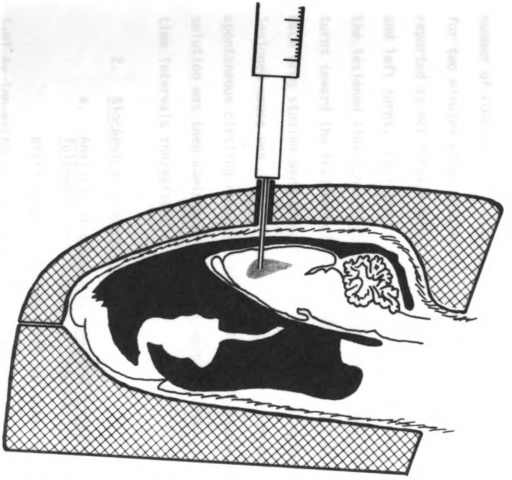

Figure 6. Sagittal view of the apparatus for making injections into the corpus striatum of the mouse brain.

mg/kg). Mice with contralateral circling responses of ten or more turns in 2 minutes were used in subsequent behavioral tests.

The circling behavior of 6-hydroxydopamine-lesioned mice was quantified by placing the mouse in a 3 liter beaker housed in a sound-attenuating box. The box was illuminated from below and the behavior was observed through a window in the top of the box. The number of complete 360° rotations to the left or right was recorded for two minutes after placing the mouse in the beaker. The results, reported as net turns/2 min., represent differences between right and left turns. Positive (+) scores indicate net turns away from the lesioned side (contralateral); negative scores indicate net turns toward the lesioned side (ipsilateral). The time courses of each drug studied were determined in the following manner. A lesioned mouse was placed in the test beaker for 2 minutes and the spontaneous circling recorded (time=0 min.); the appropriate drug solution was then administered and the mouse retested at selected time intervals thereafter.

2. Biochemical procedures

a. Analysis of dopa, dopamine, and 5-hydroxytryptamine following ion-exchange chromatographic separation

Brain samples were analyzed for dopa, dopamine and 5-HT by ion-exchange and alumina adsorption chromatography using modifications of procedures described previously (Anton and Sayre, 1964; Laverty and Taylor, 1968; Moore and Rech, 1967; Barchas et al., 1972; figure 7). In the experiments concerning either ^{3}H-L-

Figure 7. Procedure for the analysis of dopa, dopamine and 5-hydroxytryptamine (5-HT) following ion-exchange chromatography.

This illustration outlines the method used for the separation of dopa and dopamine in both the nonisotopic and isotopic studies. In the latter studies 50 μCi [^{3}H(G)]-L-dopa was intravenously injected 15 minutes prior to sacrifice by a whole body perfusion with approximately 50 ml of saline. Subsequently one forebrain, consisting of a frontal cut just caudal to the corpora quadrigemina, was extracted with perchloric acid. The ^{3}H-dopa was analyzed by taking the 1.0 ml of alumina eluate (1). The ^{3}H-dopamine was analyzed by taking the 1.0 ml of alumina eluate (2).

In the nonisotopic studies two hemiforebrains were pooled prior to extraction with perchloric acid ($HClO_4$). The alumina eluate (1) was analyzed for dopa while the column eluate was analyzed for both dopamine and 5-HT.

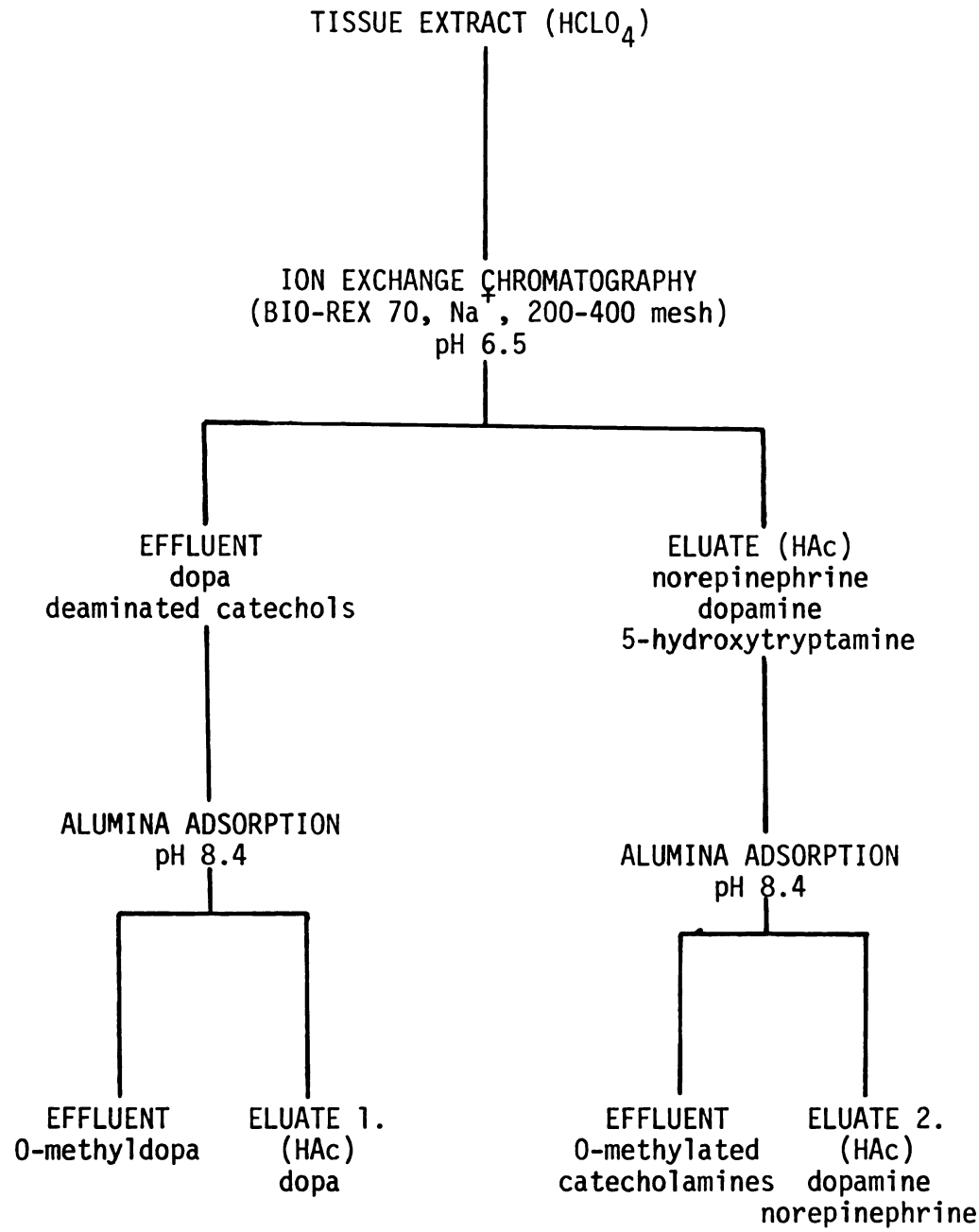

Figure 7. Procedure for the analysis of dopa, dopamine and 5-hydroxytryptamine (5-HT) following ion-exchange chromatography.

dopa or D-dopa metabolism in brain, decapitation was preceded by perfusion of the whole body of each mouse through the left ventricle with approximately 50 ml saline. Following decapitation the forebrain was isolated by a frontal cut just caudal to the corpora quadrigemina and the left and right forebrains isolated by a mid-sagittal section. Two left or two right forebrains were pooled, weighed and placed in 4.0 milliliters (ml) of 0.4 N perchloric acid (containing 0.1% EDTA). Both tissue dissection and homogenization were performed on ice. Immediately following homogenization the samples were centrifuged (10,000 x g) at 4°C for ten minutes. The supernatants were decanted into ice cold tubes containing 1.0 ml 0.1 M phosphate buffer (pH 6.5, 0.1% EDTA) and nine drops of 2 N KOH. Final adjustment to pH 6.0-6.5 was made with 0.1 N KOH. The samples were then centrifuged at 800 x g for five minutes and the supernatants placed on columns containing 3.0 centimeters of a weak cation exchange resin (Bio-Rex 70) which had been washed previously with 4.0 ml 0.1 M phosphate buffer.

The supernatant plus an additional 2.0 ml of 0.02 M phosphate buffer (pH 6.5, 1.0% EDTA) were collected as the column effluence which constitutes the dopa fraction. The dopa samples were concentrated and further purified by adsorption onto aluminum oxide (Woelm Neutral, Waters Associates, Farmington, Massachusetts) as described previously (Anton and Sayre, 1962; Drell, 1970). Following the addition of 15 drops of alumina to the column effluence, the suspension was adjusted to pH 8.0-8.5 by the addition of 0.5 ml

1 M Trizma base. The tubes were shaken for 10 minutes in an Eberbach horizontal tube shaker followed by a 5 minute centrifugation at 800 x g. After discarding the noncatechol-containing supernatant, the alumina was rinsed twice with redistilled water and then eluted with 1.0 ml of 0.2 N acetic acid. The 1.0 ml of eluate was then analyzed for dopa content by oxidation producing the fluorescent dihydroxindole derivative (Kehr et al., 1972; Lindqvist et al., 1975). The oxidation of dopa was accomplished by the sequential addition of the following reagents at 2 minute intervals: 0.2 ml 0.1 M Na_2EDTA (pH 8.0) with 1 M NaAC); 0.2 ml 0.1 N iodine; 0.2 ml alkaline sulfite; 0.2 ml 5 N HAC. The tubes were then sealed with a marble, boiled for 5 minutes and then cooled in ice. Fluorescence was determined within 15 minutes in an Aminco-Bowman spectrophotofluorometer at activating-fluorescent wavelenghts of 320-380 mμ.

Tissue blanks for the iodine oxidation procedure were determined in the following manner. The forebrain from a control mouse was carried through the separation procedure as described previously. When oxidizing, all of the reagents were measured into an empty tube. Following the addition of 5 N acetic acid, the 1.0 ml of tissue blank was added and the remaining steps carried out. The reagent blank was carried through the separation and oxidation procedure the same as a tissue sample. No difference in fluorescent readings were found between tissue and reagent blanks.

The Bio-Rex 70 columns were washed with 15 ml of 0.02 M phosphate buffer (pH 6.5, 1.0% EDTA) and twice with 3.0 ml

of redistilled water. Finally, 6.0 ml of 0.5 N acetic acid were placed on the column and the first 3.0 ml collected constituting the fraction containing both dopamine and 5-HT (Barchas et al., 1972).

The 5-HT content was analyzed by reacting 1.0 ml of the column eluate with orthophthaldialdehyde (OPT) to produce the fluorescent derivative (Curzon and Green, 1970; Haubrich and Denzer, 1973; Atack and Lindqvist, 1973; Smith et al., 1975). The fluorescent product of 5-HT was produced by the addition of 2.0 ml of concentrated HCl followed by 0.1 ml OPT to the tubes containing 1.0 ml sample. Subsequently the tubes were sealed with marbles, heated at 97-98°C for ten minutes and cooled in ice. Fluorescence was determined within 15 minutes in an Aminco-Bowman spectrophotofluorometer at activating-fluorescent wavelengths of 350-475 mμ. Tissue blanks were carried through the separation procedure like the standards and samples. During the oxidation the concentrated HCl was added to the tissue blank which were then heated and cooled in ice. The OPT was added to the tissue blank immediately before reading the fluorescence.

Dopamine content was analyzed by taking an additional 1.0 ml aliquot from the column eluate. To these samples were added 0.2 ml of 1.0 M acetate buffer (pH 8.0, 0.1 M EDTA) and the pH adjusted to pH 6.5 with 1 N NaOH, 0.1 N NaOH and 0.1 N HCl. Subsequently, the tubes were oxidized as described previously for dopa with one exception. The 0.2 ml of 1.0 M acetate buffer (pH 8.0,

0.1 M EDTA) was omitted from the first step of the timed sequence. The tissue and reagent blanks were determined as described for dopa.

Recoveries for standards of dopa, 5-HT and dopamine expressed as the mean ± one standard error, were 30.0±1.65%; 61.3± 5.5% and 88.6±4.3%, respectively. The unknown tissue concentrations were determined from these standards.

Groups of unilaterally-lesioned mice to be used in biochemical studies were sacrificed at least ten days after injection of 6-hydroxydopamine and at least three days after a test dose of apomorphine.

b. Separation of ^{3}H-dopa and metabolites

Mice were injected intraperitoneally with different AAAD inhibitors or saline 30 minutes prior to an intravenous injection of 50 μCi of ^{3}H-L-dopa in 0.25 ml of saline. Fifteen minutes later each mouse was perfused with saline through the left ventricle and the forebrain taken for analysis of ^{3}H-dopa and ^{3}H-dopamine. The biochemical separation of ^{3}H-dopa from ^{3}H-dopamine was accomplished by the detailed procedure given above (figure 7) with the following changes. The column eluate containing ^{3}H-dopamine was concentrated by adsorption onto aluminum oxide with subsequent elution as described previously for dopa. Although this fraction is referred to as ^{3}H-dopamine, it may also contain small amounts of ^{3}H-norepinephrine. The alumina eluates (10 ml) for both dopa and dopamine were placed in 10 ml Phase Combining System (PCS) (Amersham/ Searle, Arlington Heights, Illinois) and disintegrations

per minute quantified by using an external standard ratio of 35.8 in a Beckman LS-100 liquid scintillation system.

c. Analysis of 5-hydroxyindole acetic acid (5-HIAA), 5-hydroxytryptamine (5-HT), norepinephrine and dopamine following organic extraction

Brain samples were analyzed simultaneously for norepinephrine, dopamine, 5-HIAA and 5-HT by organic extraction using modifications of procedures described previously (Curzon and Green, 1970; Haubrich and Denzer, 1973; figure 8). Following decapitation, the forebrain was isolated by a frontal cut just caudal to the corpora quadrigemina and the left and right forebrains isolated by a midsagittal section. Two left or two right forebrains were pooled, weighed and placed in 4.0 ml of acidified butanol (10 mM HCl), (containing 0.1% EDTA). Both tissue dissection and homogenization were performed on ice. Following homogenization the tubes were kept on ice for 20 minutes and then centrifuged (10,000 x g, 9500 rpm) for 10 minutes at 4°C. The supernatants combined with 10 ml of heptane and 2.0 ml 0.01 N HCl were shaken for 5 minutes and centrifuged at 1000 x g for ten minutes. The organic phase combined with 1.0 ml 10 mM Tris HCl (pH 7.0) was shaken for 5 minutes and centrifuged at 1000 x g for 5 minutes. The following reagents were added sequentially to 0.5 ml of the supernatant: 0.05 ml 1% cysteine, 1.0 ml concentrated HCl; 0.05 ml OPT (made fresh in methanol) and 0.05 ml 0.2% periodate solution. These samples were kept at room temperature for 30 minutes, boiled for 10 minutes and cooled in ice. Tissue blanks were prepared by

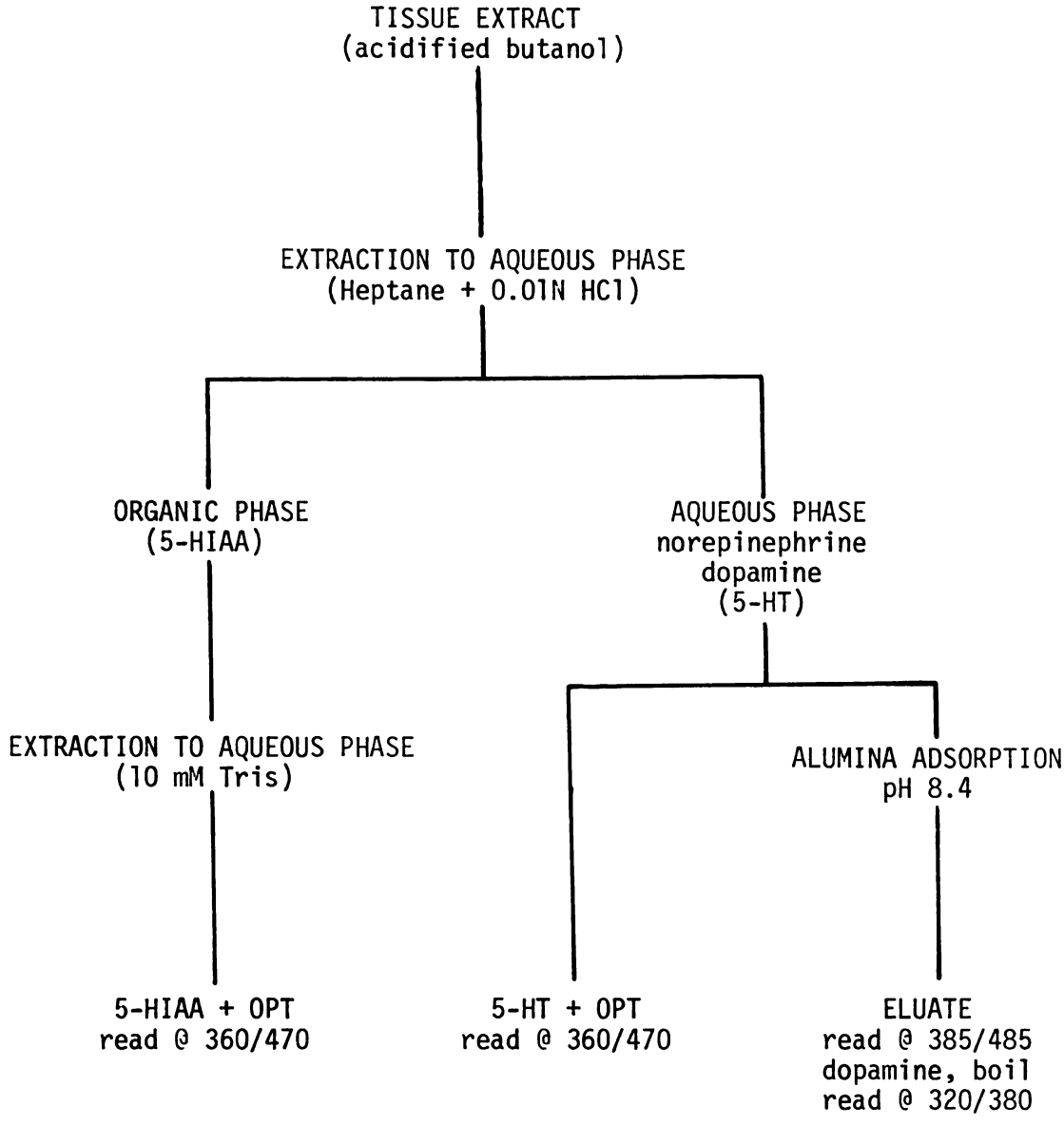

Figure 8. Procedure for the analysis of 5-hydroxyindole acetic acid (5-HIAA), 5-hydroxytryptamine (5-HT), norepinephrine and dopamine following organic extraction.

omitting the OPT until just before the fluorescence was determined. Fluorescence was determined in an Aminco-Bowman spectrophotofluorometer at activating-fluorescent wavelengths of 360-470 mμ.

The 5-HT content was analyzed by reacting 0.2 ml of the aqueous phase from the heptane mixture with 0.8 ml OPT (in 10 N HCl). The samples were heated for 15 minutes at 95°C and the fluorescence read in an Aminco-Bowman spectrophotofluorometer at activating-fluorescent wavelengths of 360-470 mμ. Tissue blanks were again prepared by adding the OPT just prior to the fluorescence reading.

The norepinephrine and dopamine concentration were quantified following alumina adsorption of 1.0 ml of the aqueous phase from the heptane mixture. Ten drops of aluminum oxide were added to 1.0 ml of the aqueous phase and the suspension shaken for 10 minutes in an Eberbach horizontal tube shaker followed by a 5 minute centrifugation at 1000 x g. After discarding the noncatechol-containing supernatant, the alumina was rinsed twice with redistilled water and then eluted with 1.0 ml of 0.2 N acetic acid. The oxidation was accomplished by the sequential addition of the following reagents at 2 minute intervals: 0.2 ml 1.0 M acetate buffer (pH 8.0, 0.1 M EDTA); 0.2 ml 1 N iodine; 0.2 ml alkaline sulfite; 0.2 ml 0.5 N acetic acid. The samples were then left at room temperature for 60 minutes and the fluorescence due to norepinephrine read at activating-fluorescent wavelengths of 385-485 mμ. Tissue blanks were prepared by adding all reagents to an empty tube. After the 5

N acetic acid was added to the empty tube, the tissue blank was combined with reagents and mixed well.

After reading the fluorescence for norepinephrine the samples were poured back into glass tubes, sealed with a marble, boiled for 5 minutes, and then cooled in ice. The fluorescence due to dopamine was read at activating-fluorescent wavelengths of 320-380 mμ. After subtracting the blank values the concentrations of 5-HIAA, 5-HT, dopamine and norepinephrine were calculated directly from the regression line of the standards.

3. Statistical analysis

a. Biochemical experiments

The data are reported as the mean ± one standard error of the mean (S.E.) obtained from the indicated number of experiments. Statistical comparisons were made with the Student's "t"-test for unpaired samples. P values of less than .05 were considered to be statistically significant (Goldstein, 1964).

b. Turnover rate comparisons

Linear regression analysis was performed by the method of least squares. The student's "t" test was used to test the parallelism of the two slopes.

c. Behavioral studies

The data are reported as the mean ± the standard error of the mean (S.E.). The same groups of lesioned mice were

used when comparisons between lesioned mice were made in one graph. Due to large differences in the variances, the Mann-Whitney U test was used for statistical determinations. Comparisons were made between the means of drug treated mice and the means of saline injected lesioned mice for the corresponding time point after injection. P values of less than 0.05 were considered indicative of statistical significance (Goldstein, 1964).

B. Materials

1. Buffers

0.1 M phosphate, pH 6.5-13.8 gm $NaH_2PO_4 \cdot H_2O$ in 900 ml 0.1% EDTA. Adjust pH to 6.5 with 5 N NaOH and q.s. to 1000 ml.

0.02M phosphate, 0.1% EDTA, pH 6.5-2.0 gm $NaH_2PO_4 \cdot H_2O$ in 800 ml 0.1% EDTA. Adjust pH to 6.5 with 5 N NaOH and q.s. to 1000 ml.

1.0 M acetate, 0.1 M EDTA, pH 8.0-3.72 gm Na_2EDTA in 80 ml hot redistilled water. Add 13.6 gm $NaC_2H_3O_2 \cdot 3H_2O$ to the cooled solution. Adjust pH to 8.0 with 5 N and 2 N NaOH and q.s. to 100 ml.

0.2 M acetate, pH 8.4-27.2 gm $NaC_2H_3O_2 \cdot 3H_2O$ (16.4 gm $NaC_2H_3O_2$, anhydrous)

1.0 M Trizma base-3.025 gm Trizma base (Tris [Hydroxyl-methyl] aminomethane, Sigma Chemical Co.) in 250 ml redistilled water

10 mM Tris-HCl, pH 7.0 0.24 g Trizma base in 180 ml H_2O and adjust to pH 7.0 with 2.0 and 1.0 N HCl and q.s. to 200 ml.

2. Reagents

Disodium ethylene diamine tetracetic acid (EDTA) 0.1% w/v (Sigma Chemical Co., St. Louis, Missouri) 1 gm Na_2EDTA in 1000 ml redistilled water-warm.

Ortho-phthaldehyde (OPT) (Sigma) 1 mg OPT/2 ml methanol (Fischer, Fairland, New Jersey) prepared immediately after oxidation.

Ortho-phthaldialdehyde (OPT in 10 N HCl) (Sigma) 12.5 mg OPT in 50 ml of 10 N HCl prepared 4-8 hours before use.

Cysteine 1%, 290 mg L-cysteine hydrochloride·H_2O (Aldrich Chemical Company, Milwaukee, Wisconsin) in 20 ml redistilled water.

Periodate solution 0.2%, 4 mg periodate $NaIO_4$ (Mallinckrodt) in 20 ml redistilled water.

Iodine 0.1 N (Mallinckrodt, St. Louis, Missouri) 1.27 gm in 100 ml 100% ethanol.

Alkaline sulfite 0.5 ml Na_2SO_3 soln + 4.5 ml 5 N NaOH, Na_2SO_3: 2.5 gm Na_2SO_3 (Fischer) in 10 ml redistilled water.

Resin Bio-Rex 70, 200-400 mesh, sodium form (Bio-Rad Laboratories, Richmond, California).

Methylcellulose (Fischer) 1.0%-10 gm in 1000 ml distilled hot water, stir and leave in cold for 12 hrs.

Sodium Metabisulfite (Baker) 0.01%-100 mg in 1000 ml distilled water.

Aluminum Oxide (Woehm neutral, Eschwege, Germany).

Acidified butanol (10 mM HCl) 0.85 ml of concentrated HCl/liter butyl alcohol (Matheson Coleman and Bell) or 0.4 ml concentrated HCl/pint butyl alcohol.

Potassium hydroxide, 10 N KOH (Fischer) 56 gm KOH in 100 ml redistilled water.

Sodium hydroxide, 5 N NaOH (Baker, Chemical Company, Phillipsburg, New Jersey) 50 gm NaOH in 250 ml cool redistilled water.

Perchloric acid, 0.4 N $HClO_4$ (Mallinckrodt) 3.54 ml of 70% $HClO_4$ q.s. 100 ml redistilled water.

Acetic acid, 5 N HAc (Mallinckrodt) 28.8 ml of glacial HAc q.s. 100 ml redistilled water.

Hydrochloric acid, 2 N HCl (Baker) 190 ml concentrated HCl q.s. to 100 ml redistilled water.

3. Standards

Norepinephrine 1 mg/ml solution

18.87 mg norepinephrine bitartrate (Sigma) in 10 ml 0.2 N HAc.

Dopamine 1 mg/ml solution

12.35 mg dopamine HCl (Sigma) in 10 ml 0.2 N HAc.

5-HT 100 mg/ml solution

2.30 mg 5-hydroxytryptamine creatinine sulfate monohydrate (Aldrich) in 10 ml 0.2 N HAc.

DOPA 1 mg/ml solution

10 mg L-dihydroxyphenylalanine (Sigma) in 10 ml 0.1 N HCl.

4. Resin preparation

Place 0.5 lb of Bio-Rex 70 (200-400 mesh, Na^+) in a liter bottle and fill with distilled water. Adjust pH to 6.5 with concentrated HCl under stirring. Hand shake, wait about 30 minutes until supernatant clear and decant the water from resin. Repeat following procedure eight times: add water, hand shake, wait and decant. Finally add 500 ml 0.1 M phosphate buffer (pH 6.5, 0.1% EDTA) to the washed Bio-Rex, keep refrigerated.

5. Alumina purification

One half bottle of Woelm aluminum oxide (distributed by Waters Assoc. Inc., Farmington, Massachusetts) is placed in a liter bottle. A suspension of the alumina is shaken repeatedly with distilled water until supernatant becomes clear. Add 200 ml of 2.0 N HCl, shake for 30 minutes and decant the supernatant. Wash ten times with distilled water. Wash once with 0.2 N HAc and once with 0.02 N Na acetate (pH 8.4). Wash with distilled water ten times and then redistilled water five times. Store the alumina in a glass stoppered bottle containing sufficient water to thoroughly wet the alumina.

6. Drugs

6-Hydroxydopamine hydrobromide was obtained initially from Regis Chemical Company (Chicago, Illinois) and later from Sigma Chemical Company (St. Louis, Missouri). Apomorphine HCl, obtained from Eli Lilly and Company (Indianapolis, Indiana) was dissolved in 0.01% sodium metabisulfite. L-α-methyldopa hydrazine (HMD or MK 486), a gift of Dr. C. Stone, Merck Sharp & Dohme, was prepared for injection as a suspension in 1.0% methylcellulose. L- and D-dopa were obtained from both Nutritional Biochemicals Corporation (NBC; Cleveland, Ohio) and Sigma Chemical Company (St. Louis, Missouri) and were suspended in 1.0% methylcellulose. Meta-hydroxybenzylhydrazine (NSD 1015) was obtained from Aldrich Chemical Company (Milwaukee, Wisconsin). Methoxyflurane (Metofane) was obtained from Pitman-Moore, Inc. (Washington Crossing, New Jersey). 4-bromo-3-hydroxybenzyloxyamine dihydrogen phosphate (NSD 1055, brocresine) was a gift from Dr. Leon Ellenbogen at Lederle Laboratories (Pearl River, New York). N-(DL-seryl)-N'-(2,3,4-trihydroxybenzyl) hydrazine (Ro44602, Benserazid) was a gift from Dr. W. E. Scott at Hoffman-LaRoche, Inc. (Nutley, New Jersey). L-dopa for the optical purity studies was obtained from Calbiochem (Elk Grove Village, Illinois). [^{3}H(G)]-L-3,4-dihydroxyphenylalanine (11.5 Ci/mmole, ^{3}H-dopa) was purchased from New England Nuclear (Boston, Massachusetts). Pargyline HCl (Eutonyl) was obtained from Abbott Laboratories (Chicago, Illinois). 5,7-Dihydroxytryptamine creatinine sulfate was purchased from Regis Chemical Company (Chicago, Illinois).

Except where indicated all drugs were dissolved in saline. The drugs were weighed as the salts, prepared immediately before use and administered in a volume of 1 ml/100 gm body weight.

RESULTS

A. Behavioral rotation studies

1. Dose-response relationship between D- and L-dopa

Previous studies (Ungerstedt, 1971a,b; Von Voigtlander and Moore, 1973; Fuxe and Ungerstedt, 1974; Mendez et al., 1975) have demonstrated that L-dopa induces contralateral circling in rodents with unilateral nigrostriatal pathway lesions. In order to provide further insight into the mechanism of action of L-dopa, the behavioral and biochemical properties of L-dopa were compared to those of its enantiomer, D-dopa.

A comparison of the L- and D-dopa-induced circling behavior in mice with unilateral 6-hydroxydopamine striatal lesions is presented in figure 9. Prior to the testing of dopa, groups of ten lesioned mice were selected for equivalent contralateral circling responses to apomorphine; the means of the four groups of mice used in figure 9 were: +17.80±2.64; +16.90±2.28; +17.90± 2.30; +18.60±1.91. There were no significant differences between the latter apomorphine treatment groups as determined by the unpaired Student's t-test ($p<.05$). In figure 9 the solid symbols indicate those values different from methylcellulose-treated 6-hydroxydopamine-lesioned controls as determined by the Mann-Whitney U test ($p<.05$).

As demonstrated in figure 9A, L-dopa reversed the initial ipsilateral (negative) circling at time 0 minutes to produce contralateral (positive) circling. Administration of increasing doses of L-dopa (5, 10 and 20 mg/kg) resulted in a dose-related increase in the contralateral circling (figure 9A).

If the contralateral circling induced by L-dopa is mediated by the formation of dopamine then the administration of D-dopa should not induce contralateral circling since it is not metabolized by the brain to dopamine (Shindo et al., 1973). However, figure 9B demonstrates that the intraperitoneal administration of D-dopa unexpectedly produced a marked increase in contralateral circling. Administration of D-dopa at 50, 100 and 200 mg/kg resulted in a dose-related increase in the circling away from the lesioned side. Furthermore, approximately ten times as much D-dopa had to be administered to produce contralateral circling rates equivalent to those produced by L-dopa (i.e., 10 mg/kg of L-dopa and 100 mg/kg of D-dopa). It is also noteworthy that following the high dose of D-dopa the duration of circling is greater when compared to the L-dopa-induced circling (compare figures 9A and B). The contralateral circling following D-dopa (200 mg/kg) is significantly elevated at 45 and 60 minutes in contrast to the L-dopa-induced circling which has subsided at these time points.

Figure 9. Effects of L- and D-dopa on circling behavior in mice with unilateral 6-hydroxydopamine lesions.

Seven days after injection of 6-hydroxydopamine into the corpus striatum mice were tested for apomorphine (0.25 mg/kg s.c.)-induced contralateral circling behavior. Four groups of mice having equivalent rates of apomorphine-induced circling were used in studying the L- and D-dopa-induced contralateral circling. The symbols represent the means and the vertical lines denote one standard error as determined in 10 mice. The symbols ○, △, and □ indicate values obtained with the intraperitoneal administration of 5, 10 and 20 mg/kg of L-dopa and 50, 100 and 200 mg/kg of D-dopa. The standard error is indicated by the vertical lines on each symbol except when the value is less than the radius of the symbol. Solid symbols indicate values that are significantly different from those mice injected with 1% methylcellulose ($p<.01$).

Negative values on the ordinate represent circling toward the lesioned side (ipsilateral) whereas positive values represent circling away from the lesioned side (contralateral).

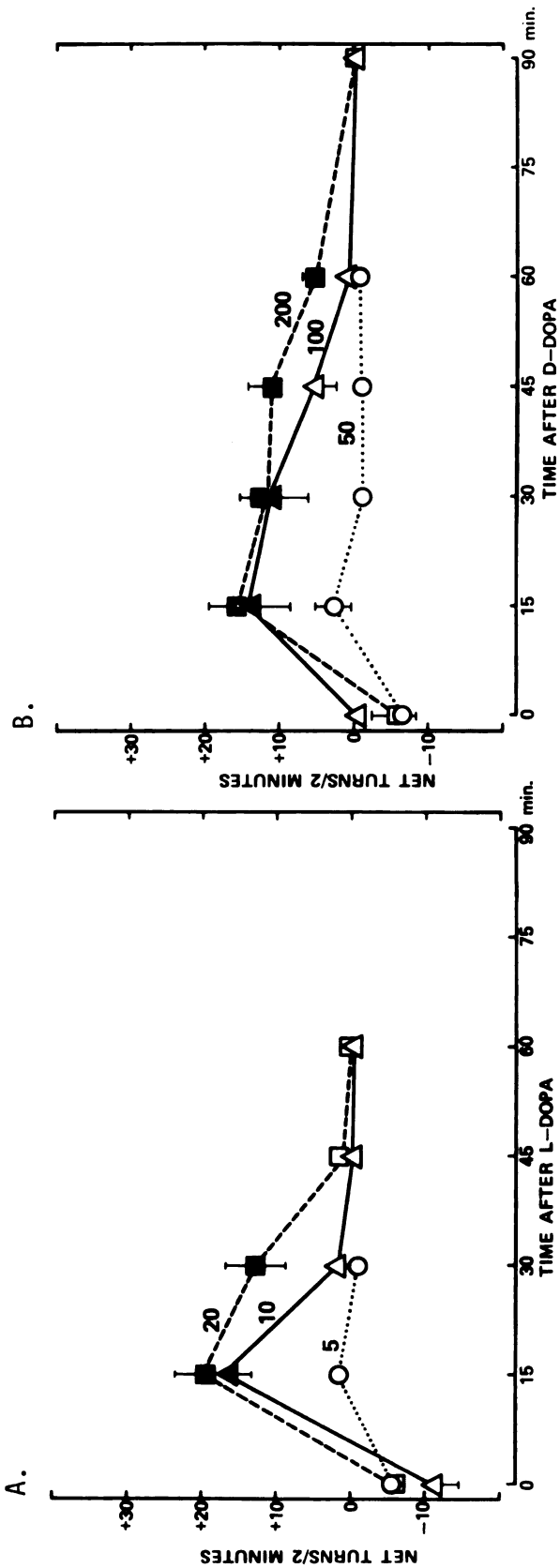

Figure 9. Effects of L- and D-dopa on circling behavior in mice with unilateral 6-hydroxydopamine lesions.

2. Central decarboxylase inhibition and dopa-induced contralateral circling

a. Ro44602

Previous studies by Ungerstedt (1971) demonstrated that inhibition of the AAAD activity by high doses of Ro44602 would delay the contralateral circling response of lesioned rats to L-dopa. In order to determine if the delay in circling was related to decreased dopamine formation, the D-dopa-induced contralateral circling was studied following Ro44602.

A comparison of the circling response to L- or D-dopa following Ro44602 is depicted in figure 10. The same group of lesioned mice was used for making comparisons within each graph. Subcutaneous administration of L-dopa (10 mg/kg) produced the expected increase in contralateral circling (figure 10A). Following pretreatment with Ro44602, there was an initial delay followed by a prolonged period of circling as has been demonstrated previously (Ungerstedt, 1971b).

The subcutaneous administration of D-dopa (100 mg/kg) did induce contralateral circling but the amount of circling was not as large as that following intraperitoneal administration. When the D-dopa injection followed pretreatment with Ro44602, the contralateral circling was initially blocked for 60 minutes. At 90 minutes an increase in contralateral circling occurred and was still elevated at 360 minutes after D-dopa (figure 10B). Thus, the D-dopa-induced contralateral turning was unexpectedly delayed by an

Figure 10. Effects of peripheral and central aromatic-L-amino acid decarboxylase (AAAD) inhibition by Ro44602 on the contralateral circling behavior induced by L- and D-dopa.

Apomorphine (0.25 mg/kg s.c.) was used to test mice lesioned one week previously with 6-hydroxydopamine. Mice with at least 10 net contralateral turns/2 minutes were used in this behavioral study. The comparison within each graph is made between the same group of lesioned animals. L-Dopa (10 mg/kg) or D-dopa (100 mg/kg) were injected subcutaneously 30 minutes after the intraperitoneal injection of saline (O) or Ro44602 (800 mg/kg, Δ). The symbols represent the means and the vertical lines denote one standard error as determined in 7 mice for the L-dopa study and 12 mice for the D-dopa study. Solid symbols indicate values that are significantly different from mice injected with 1% methylcellulose ($p<.05$).

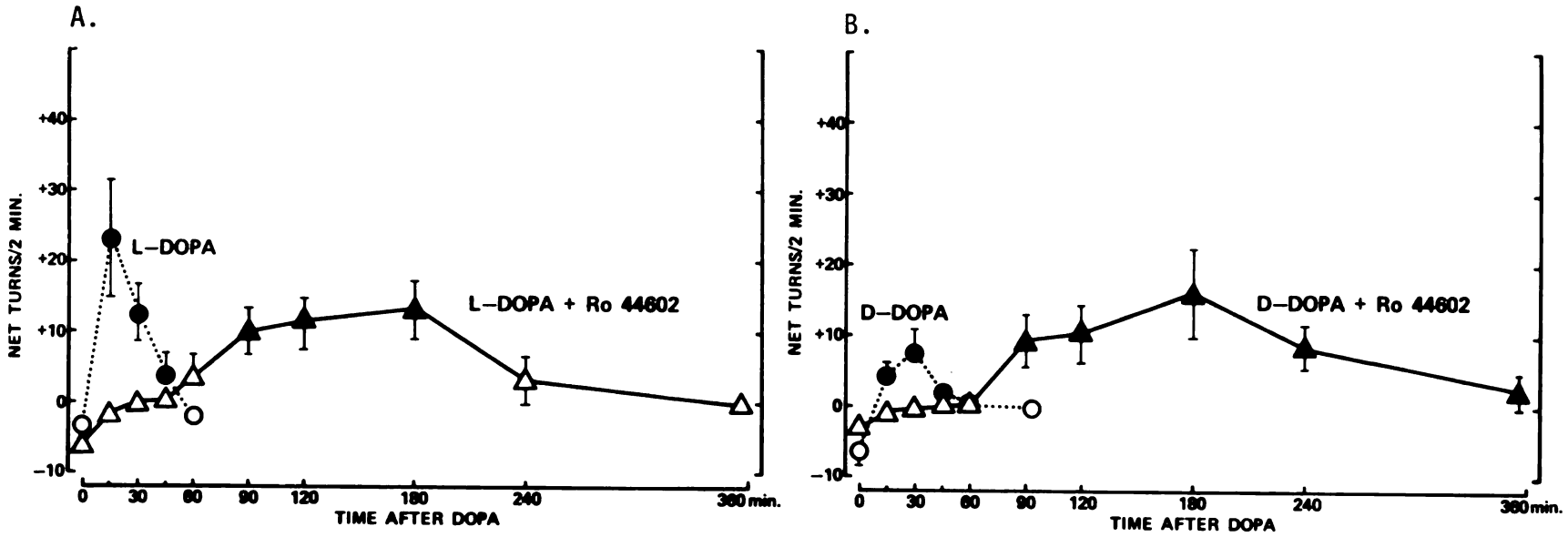

Figure 10. Effects of peripheral and central aromatic-L-amino acid decarboxylase (AAAD) inhibition by Ro44602 on the contralateral circling behavior induced by L- and D-dopa.

inhibitor of L-amino acid decarboxylation in the central nervous system. This initial delay of dopa-induced circling by Ro44602 may be attributable to a pharmacological action other than inhibition of brain AAAD activity. Therefore another inhibitor of brain AAAD activity, NSD 1015 was administered as a pretreatment to the dopa-induced contralateral circling.

b. NSD 1015

A comparison of circling behavior induced by L-dopa, apomorphine and D-dopa following AAAD inhibition by NSD 1015 is presented in figure 11. The comparison within each graph is made between the same group of lesioned mice. Figure 11 demonstrates that inhibition of central and peripheral AAAD activity completely abolished the L-dopa-induced contralateral circling. The L-dopa-induced contralateral circling did not recur at later time intervals as evidence by -0.83±0.72 (mean ± 1 S.E.) turns/2 minutes at 180 minutes after L-dopa administration. D-Dopa, which has been previously considered not to be metabolized to dopamine, also did not induce contralateral circling following inhibition of the enzyme AAAD (figure 11A).

One alternative mechanism through which NSD 1015 might be inhibiting the contralateral circling is via blockade of dopaminergic postsynaptic receptors. In an effort to test this hypothesis, the contralateral circling response to a direct-acting dopaminergic agonist, apomorphine, was studied following pretreatment with NSD

Figure 11. Effects of peripheral and central aromatic-L-amino acid decarboxylase (AAAD) inhibition by NSD 1015 on the contralateral circling behavior induced by L-dopa, apomorphine and D-dopa.

Seven days after the 6-hydroxydopamine lesions mice were tested for apomorphine (0.25 mg/kg s.c.)-induced contralateral circling and those mice with more than ten turns/2 minutes were used in this experiment. The comparison within each graph is made between the same group of lesioned animals. Mice received an intraperitoneal injection of saline (O) or NSD 1015 (100 mg/kg, ●) 30 minutes before the subcutaneous injection of L-dopa (10 mg/kg), apomorphine (0.25 mg/kg) or D-dopa (100 mg/kg). The symbols represent the mean and the vertical lines denote one standard error as determined in 12 mice for the L-dopa and apomorphine studies and 4 mice for the D-dopa studies.

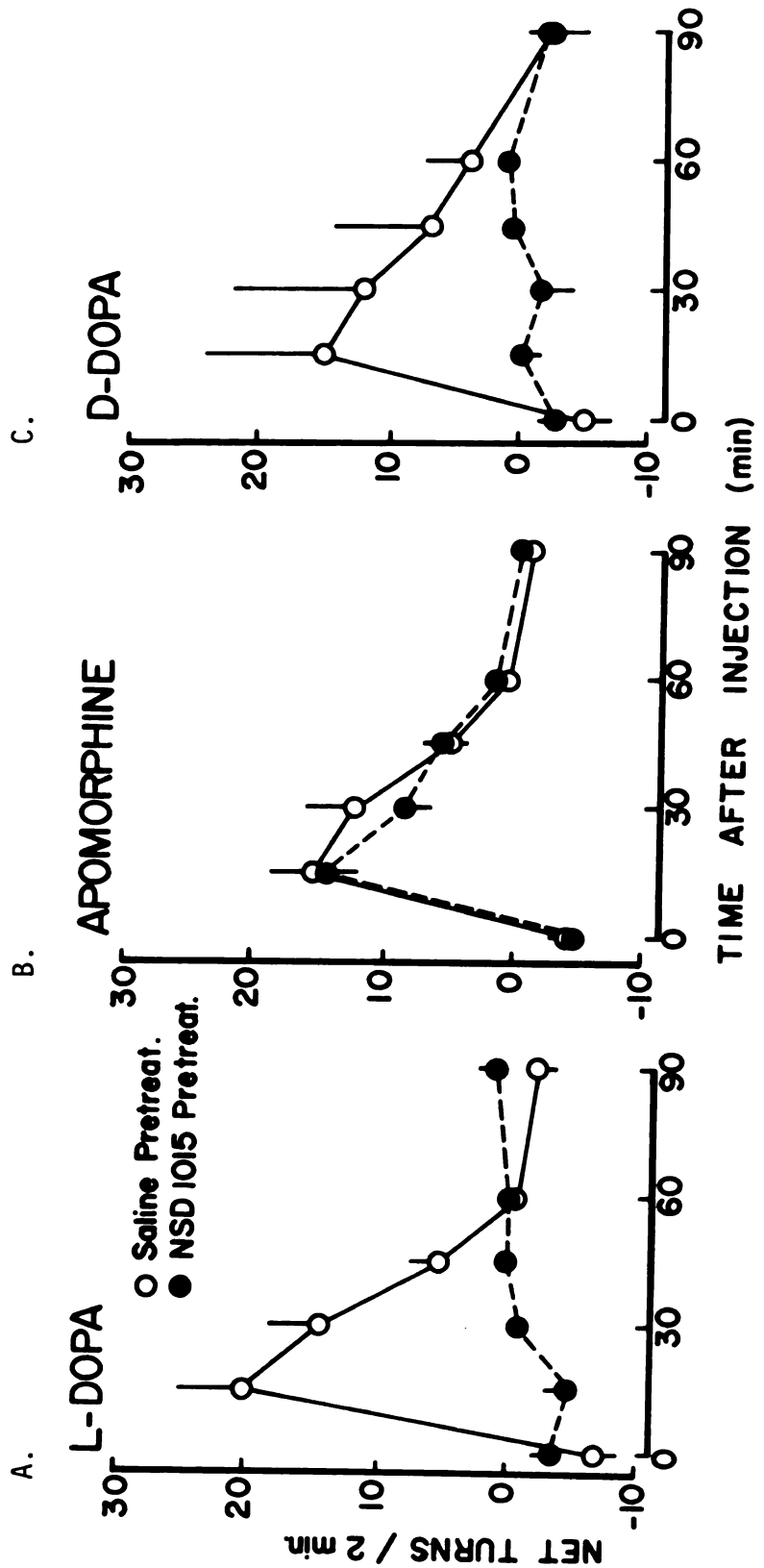

Figure 11. Effects of peripheral and central aromatic-L-amino acid decarboxylase (AAAD) inhibition by NSD 1015 on the contralateral circling behavior induced by L-dopa, apomorphine and D-dopa.

1015. Figure 11B demonstrates that NSD 1015 did not alter the contralateral circling response to apomorphine, suggesting that the AAAD inhibitor has no dopaminergic receptor blocking properties.

3. Peripheral decarboxylase inhibition

a. Hydrazinomethyldopa (HMD)

Because of the demonstrated similarity in the central action of the two isomers, it was of interest to compare the role of peripheral decarboxylation in the metabolism of D- and L-dopa. For this purpose HMD, an AAAD inhibitor which does not enter the brain, was given as a pretreatment one hour before the injection of dopa.

A comparison of the contralateral circling responses to L-and D-dopa following inhibition of peripheral AAAD activity by HMD is presented in figure 12. The contralateral circling response to L-dopa was prolonged by pretreatment with HMD (figure 12A) as also demonstrated by Mendez et al. (1975). The latter effect was expected but the contralateral circling response to D-dopa was also prolonged and enhanced by HMD (figure 12B) which blocks only the decarboxylation of L-amino acids.

b. NSD 1055 (brocresine)

NSD 1055 has been utilized by various investigators (Levine and Sjoerdsma, 1964; Robson, 1971; Roberge et al., 1972; Howse and Matthews, 1973) as a central and peripheral AAAD inhibitor.

Figure 12. Effects of peripheral aromatic-L-amino acid decarboxylase (AAAD) inhibition by hydrazinomethyldopa (HMD) on the contralateral circling behavior induced by L- and D-dopa.

Seven days after the injection of 6-hydroxydopamine into the corpus striatum, mice were given a test dose of apomorphine (0.25 mg/kg s.c.). Those mice with more than ten turns/2 minutes were used during this experiment. Mice received intraperitoneal injections of saline (O) or hydrazinomethyldopa (HMD, 25 mg/kg, Δ) one hour before the subcutaneous injection of L-dopa (10 mg/kg) or D-dopa (100 mg/kg). The symbols represent the means and the vertical lines denote one standard error as determined in 12 mice. Solid symbols indicate values that are significantly different from mice injected with 1% methylcellulose ($p<.05$).

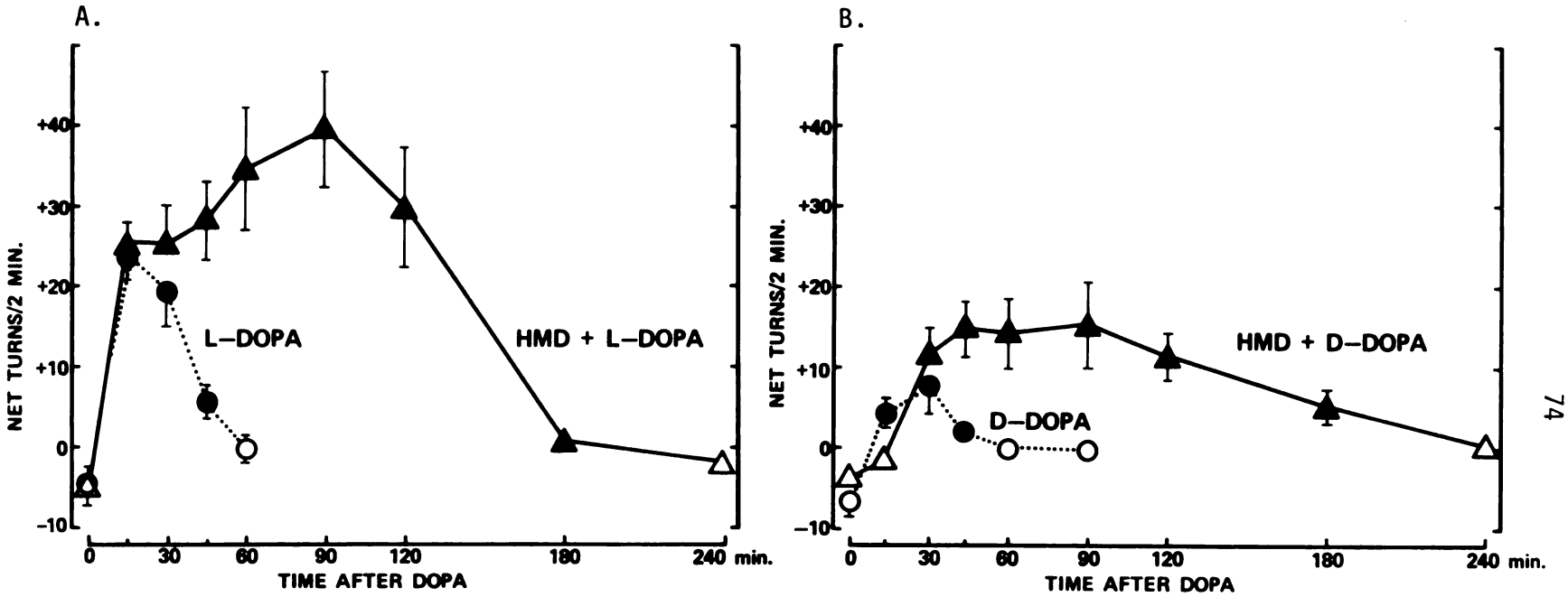

Figure 12. Effects of peripheral aromatic-L-amino acid decarboxylase (AAAD) inhibition by hydrazinomethyldopa (HMD) on the contralateral circling behavior induced by L- and D-dopa.

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Seven days after the injection of 6-hydroxydopamine into the corpus striatum, mice were given a test dose of apomorphine (0.25 mg/kg s.c.). Those mice with more than ten turns/2 minutes were used during this experiment. Mice received intraperitoneal injections of saline (O) or hydrazinomethyldopa (HMD, 25 mg/kg, Δ) one hour before the subcutaneous injection of L-dopa (10 mg/kg) or D-dopa (100 mg/kg). The symbols represent the means and the vertical lines denote one standard error as determined in 12 mice. Solid symbols indicate values that are significantly different from mice injected with 1% methylcellulose ($p<.05$).

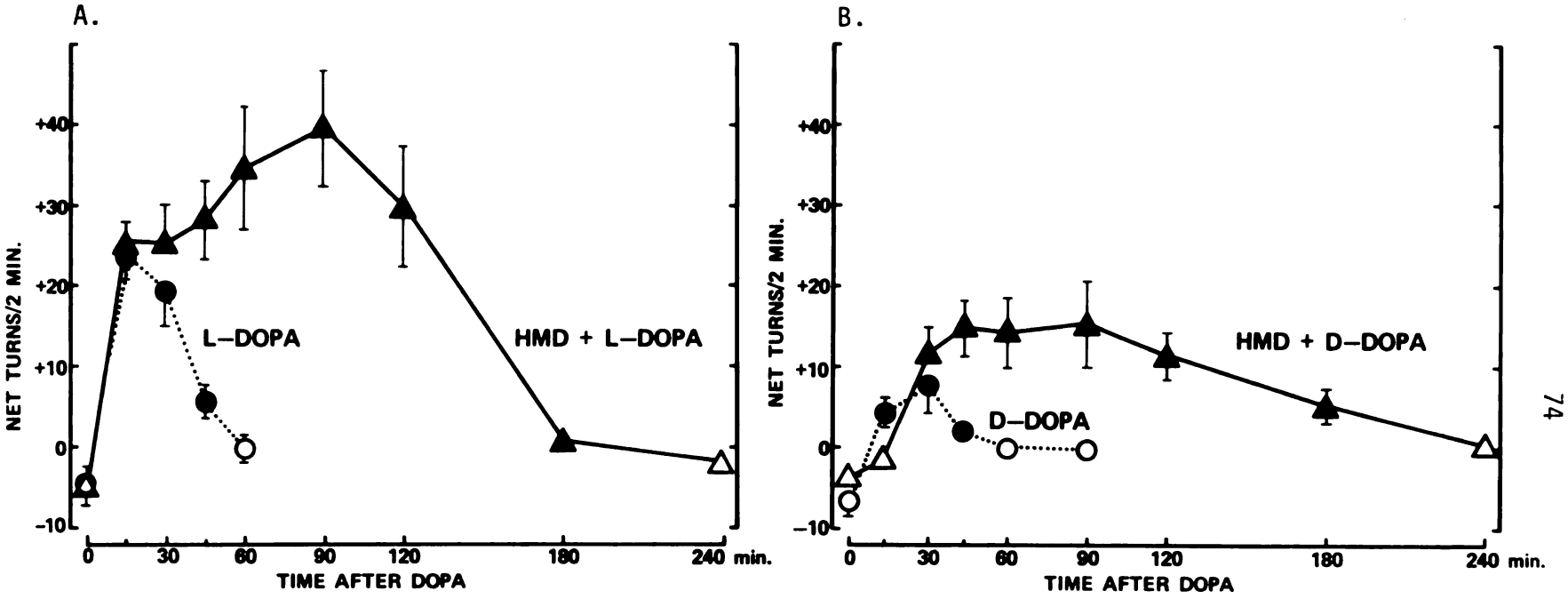

Figure 12. Effects of peripheral aromatic-L-amino acid decarboxylase (AAAD) inhibition by hydrazinomethyldopa (HMD) on the contralateral circling behavior induced by L- and D-dopa.

Thus, it was of interest to study the effect of NSD 1055 on the contralateral circling induced by L- and D-dopa.

The L- and D-dopa-induced contralateral circling responses following pretreatment with saline or NSD 1055 30 minutes prior to dopa did not inhibit the contralateral circling induced by either isomer (Table 1). Furthermore, the contralateral circling induced by L- and D-dopa was of greater duration following NSD 1055 than the corresponding saline controls. Data from these behavioral tests suggests that NSD 1055 resembles the peripheral AAAD inhibitor, HMD, more than the central AAAD inhibitors, Ro44602 and NSD 1015.

B. Resolution of enantiomeric purity of D- and L-dopa

In an effort to determine if the contralateral circling response to D-dopa was due to an impurity of L-dopa in our samples, D- and L-dopa obtained from different sources were analyzed for their optical purity (Corts and Koekkoek, 1971; Appleby and Mitchell, 1971).

The specific rotation of different samples of L- and D-dopa are presented in Table 2 with the specific rotation values from the literature. The data presented in the table were collected during two separate experiments.

The specific rotations of the L-dopa samples were comparable to those reported in the literature. The important point to be emphasized is that the D-dopa samples when compared to the literature appeared to be optically pure (i.e., D-dopa values +12.00, +11.95 and +12.55; literature values: 12.25 and 12.10; Table 2).

Table 1. Effects of NSD 1055 (brocresine) on the contralateral circling behavior induced by L- and D-Dopa.

TIME (MINUTES) AFTER DOPA ADMINISTRATION	L-DOPA		D-DOPA	
	SALINE	NSD 1055	SALINE	NSD 1055
15	+24.1±3.6*	+13.7±3.6*	+7.5±2.0*	+28.5±4.9*
30	+13.9±3.9*	+20.4±7.3*	+11.5±4.6*	+20.6±5.1*
45	+0.9±1.5	+7.6±2.6*	+3.8±1.5*	+22.6±5.7*
60	-1.1±0.6	-0.2±0.8	+0.9±0.9	+16.6±3.9*

Seven days after the injection of 6-hydroxydopamine into the corpus striatum, mice were tested for apomorphine (0.25 mg/kg, s.c.)-induced circling behavior. Those mice with more than ten contralateral turns/2 minutes were used during this experiment. Mice were given intraperitoneal injections of L-dopa (10 mg/kg) or D-dopa (100 mg/kg) 30 minutes after the intraperitoneal injection of saline or NSD 1055 (100 mg/kg). Different groups of mice were used in each study with L- or D-dopa. Values represent the mean ± 1 S.E. of twelve experiments.

*Significantly different from control as determined by the Mann-Whitney U test ($p<.05$).

Table 2. Specific rotation of different samples of L- and D-dopa.

	SPECIFIC ROTATION	
	EXPERIMENT 1	EXPERIMENT 2
INDIVIDUAL SAMPLES		
L-Dopa (Sigma)	-11.80	-11.60
L-Dopa (NBC)	-12.15	-11.65
L-Dopa (Calbiochem)	-11.65	-11.55
D-Dopa (Sigma)	+12.00	+11.95
D-Dopa (NBC)	+12.55	------
COMBINED SAMPLES (Sigma)		
99% D-Dopa + 1% L-Dopa	------	+11.80
98% D-Dopa + 2% L-Dopa	------	+11.45
97% D-Dopa + 3% L-Dopa	------	+11.65
96% D-Dopa + 4% L-Dopa	------	+11.00
95% D-Dopa + 5% L-Dopa	------	+11.00
90% D-Dopa + 10% L-Dopa	+9.95	+9.95
80% D-Dopa + 20% L-Dopa	+6.95	+7.05
REPORTED VALUES		
Corts and Koekkoek, 1971	-12.25	
Appleby and Mitchell, 1971	-12.10	

Experiments were performed with a Perkin Elmer 141 Polarimeter at 20°C using a mercury lamp (λ=587). Dopa was dissolved in 1 N HCl (20 mg/ml).

$$\text{specific rotation } ([\alpha]^{20}_{578}) = \frac{\text{observed rotation}}{(1 \text{ decimeter})(\text{g/ml})}$$

In order to determine the relationship between increasing amounts of L-dopa in individual D-dopa samples, a series of combined samples containing increasing concentrations of L-dopa were prepared and analyzed for changes in specific rotation. Increasing the amount of L-dopa (from 1 to 5%) contained in D-dopa samples resulted in a gradual reduction in the specific rotation.

If the response to D-dopa was due to a contaminant of L-dopa, then the D-dopa sample would have to contain approximately 10% of the L-isomer as an impurity since the dose of L-dopa essential to produce an equivalent rate of circling is one tenth the dose of D-dopa (compare D- and L-dopa dose responses in figure 9). One D-dopa sample was prepared containing 10% L-dopa. The calculated value for specific rotation changed from +12.00 and +11.95 to +9.95 emphasizing that our D-dopa samples were not contaminated with significant quantities of L-dopa (Table 2).

C. Biochemical Studies

1. ^{3}H-L-Dopa metabolism

The effects of AAAD inhibitors on the brain content of ^{3}H-dopa and ^{3}H-dopamine following systemic administration of ^{3}H-L-dopa are presented in Table 3. An intravenous injection of ^{3}H-L-dopa 15 minutes prior to sacrifice resulted in an equivalent increase in forebrain concentrations of ^{3}H-dopa and ^{3}H-dopamine in mice pretreated with saline 30 or 195 minutes before the ^{3}H-L-dopa.

Table 3. Effects of aromatic-L-amino acid decarboxylase (AAAD) inhibitors on the brain contents of ^{3}H-dopa and ^{3}H-dopamine following systemic administration of ^{3}H-L-dopa.

PRETREATMENT		TIME (MINUTES) before ^{3}H-L-dopa injection	FOREBRAIN CONCENTRATION (nCi/gram)	
			DOPA	DOPAMINE
Saline		30	8.83± 1.64	6.38±1.10
		195	8.14± 1.84	6.21±0.57
HMD	25 mg/kg	60	48.32± 7.98	19.00±1.48
		225	42.69± 7.51	20.24±4.59
Ro44602	800 mg/kg	30	92.94±13.03	0.78±0.32
		195	64.28± 4.24	15.31±2.20
NSD 1015	100 mg/kg	30	89.30±13.63	0.47±0.08
		195	66.14±15.34	9.67±1.94
NSD 1055	100 mg/kg	30	61.32± 6.93	11.22±0.66
α-Methyldopa	500 mg/kg	30	23.35± 2.43	16.25±2.65

Various AAAD inhibitors or saline were administered i.p. to mice at the indicated times prior to an intravenous injection of 50 μCi of ^{3}H-L-dopa in 0.25 ml saline. Fifteen minutes later the whole body of each mouse was perfused through the left ventricle with approximately 50 ml saline and the forebrain taken for analysis of ^{3}H-dopa and ^{3}H-dopamine. Values represent the mean ± 1 S.E. as determined in 7 mice for both saline studies and with 4 mice in each study with a decarboxylase inhibitor. All drug treatments resulted in values of ^{3}H-dopa and ^{3}H-dopamine which were statistically different ($p<.05$) from the corresponding saline controls except for the ^{3}H-dopamine concentration following pretreatment with NSD 1015 at 195 minutes.

HMD pretreatment in rats has been reported to inhibit only peripheral AAAD causing increased cerebral concentrations of ^{3}H-catecholamines following systemic ^{3}H-L-dopa (Porter et al., 1962; Bartholini and Pletscher, 1969). Administration of HMD to mice 60 or 225 minutes before intravenous ^{3}H-L-dopa resulted in a marked increase in the cerebral concentrations of both ^{3}H-dopa and ^{3}H-dopamine (Table 3), thus confirming the results of earlier studies with HMD.

Previous studies (Bartholini et al., 1967, 1969; Bartholini and Pletscher, 1968, 1969) have demonstrated that in rats Ro44602 inhibits peripheral and central AAAD activity at high doses (500 mg/kg). Pretreatment of mice with Ro44602 at 30 or 195 minutes prior to ^{3}H-L-dopa caused a large increase in the forebrain ^{3}H-dopa concentration, reflecting the inhibition of peripheral AAAD (Table 3). Furthermore, the forebrain ^{3}H-dopamine concentrations were reduced at 30 minutes, but increased at 195 minutes after Ro44602 administration. The increase in ^{3}H-dopamine concentration at 195 minutes presumably reflects a reduction in the concentration of Ro44602 in the brain below that which is necessary to inhibit cerebral AAAD activity.

NSD 1015 has been demonstrated in mice and rats to inhibit both peripheral and central AAAD activity (Bartholini and Pletscher, 1969; Carlsson and Lindqvist, 1973; Hulme et al, 1974). Pretreatment with NSD 1015 at 30 or 195 minutes prior to ^{3}H-L-dopa markedly increased forebrain ^{3}H-dopa concentrations reflecting an inhibition of peripheral metabolism (Table 3). NSD 1015 pretreatment blocked

the increase in ^{3}H-dopamine concentration at 30 minutes, but at 195 minutes the ^{3}H-dopamine concentration was not significantly different from the corresponding saline controls.

NSD 1055 (brocresine) has been reported to inhibit both the peripheral and central AAAD activity (Levine and Sjoerdsma, 1964; Roberge et al., 1972; Howse and Matthews, 1973; Robson, 1971). Although NSD 1055 administration significantly enhanced the forebrain content of ^{3}H-dopa, it did not block, but rather increased the content of ^{3}H-dopamine when compared to controls (Table 3).

α-Methyldopa has been utilized to inhibit peripheral and central AAAD activity (Smith, 1962; Fermaglich and O'Doherty, 1971; Vazquez and Sabelli, 1975). The administration of α-methyldopa (500 mg/kg) 30 minutes before ^{3}H-L-dopa significantly increased the forebrain content of both ^{3}H-dopa and ^{3}H-dopamine when compared to controls suggesting an inhibition of peripheral but not cerebral AAAD activity.

The data in Table 3 demonstrate that Ro44602 at a dose of 800 mg/kg or NSD 1015 at a dose of 100 mg/kg markedly inhibit the conversion of ^{3}H-dopa to ^{3}H-dopamine in the forebrain 30 minutes after administration. Thus, only these agents may be utilized to block the conversion of dopa to dopamine in the central nervous system.

2. D-Dopa metabolism in brain

The following experiment was performed to determine if the administration of D-dopa would elevate cerebral dopamine concentrations analogous to that seen following the L-isomer (Thut and Rech, 1972; Horst et al., 1973; and Weiss et al., 1974). A comparison of the effect of L- and D-dopa on the endogenous forebrain concentration of dopa and dopamine is presented in figure 13. After the administration of doses of L- and D-dopa that caused approximately equal rates of contralateral circling (10 mg/kg and 100 mg/kg, respectively), only L-dopa caused a significant increase in the brain content of dopamine. The accumulation in the brain of both isomers of dopa was, however, approximately the same. The preferential accumulation of the lower dose of L-dopa compared to D-dopa was not related to an artifact of the assay procedure since both L- and D-dopa had standard recovery curves which were identical.

3. Depletion of 5-hydroxytryptamine

a. L-Dopa in 6-hydroxydopamine-lesioned mice

Previous studies in animals with lesions of the nigrostriatal pathway have reported conflicting results in the extent of 5-HT depletion by L-dopa (Uretsky and Schoenfeld, 1971; Poirier et al., 1971; Lytle et al., 1972; Langelier et al., 1973). Therefore, the following study has compared the dopa-induced changes of monoamine concentrations in mice with unilateral lesions of striatal dopaminergic nerve terminals.

Figure 13. Effects of L- and D-dopa administration on the endogenous concentrations of dopa and dopamine in mouse forebrain.

The concentration of dopa and dopamine in forebrains of mice was determined following the administration of doses of L- and D-dopa which induced similar contralateral circling rates. One hour after the intraperitoneal administration of L-dopa (10 mg/kg) or D-dopa (100 mg/kg) mice were anesthetized with methoxyflurane and their bodies perfused with approximately 50 ml of saline. The forebrains were then removed and analyzed for dopa and dopamine. Mice were pretreated with hydrazinomethyldopa (HMD; 25 mg/kg i.p.) one hour prior to the dopa injections.

*Significantly different from control values ($p<0.01$).

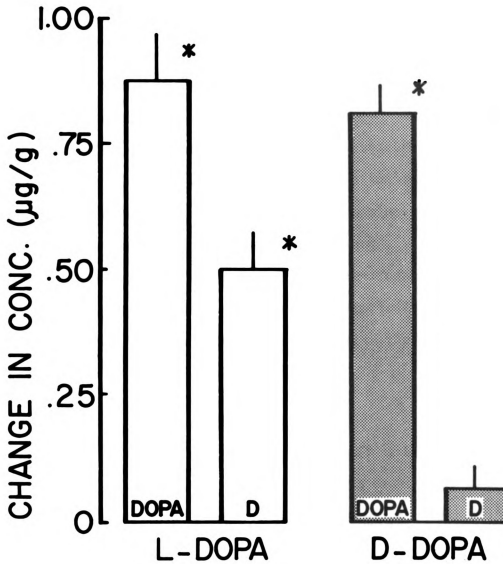

Figure 13. Effects of L- and D-dopa administration on the endogenous concentrations of dopa and dopamine in mouse forebrain.

The concentrations of dopa, dopamine and 5-HT are presented in figure 14 for control and 6-hydroxydopamine-lesioned hemiforebrains of mice. The injection of 6-hydroxydopamine into the striatum markedly reduced the dopamine concentration to 12% of control while not affecting the 5-HT or dopa concentrations. In both the non-lesioned and 6-hydroxydopamine-lesioned hemiforebrains L-dopa administration caused a dose-related increase in the concentration of dopa and dopamine with a concomitant reduction in the concentration of 5-HT. The increase in dopa concentration and the decrease in 5-HT concentration within the 6-hydroxydopamine-lesioned hemisphere were not significantly different from the changes in dopa and 5-HT concentrations observed in the nonlesioned hemiforebrain. The only significant difference between the control and lesioned hemiforebrains was a reduction in the dopamine accumulation in the latter following both doses of L-dopa (figure 14).

b. Comparison of D- and L-dopa

The depletion of forebrain 5-HT by L-dopa observed during *in vitro* studies has been suggested to require the prerequisite conversion of dopa to dopamine (Ng *et al.*, 1971, 1972a,b). In an attempt to answer this question with *in vivo* studies, the forebrain 5-HT content was determined following the administration of D-dopa.

A comparison of high doses of L- and D-dopa on the forebrain contents of dopa, dopamine, and 5-HT is given in figure 15. Administration of L-dopa (100 and 200 mg/kg) resulted in an increase in

Figure 14. Comparison of L-dopa-induced depletion of 5-hydroxytryptamine (5-HT) in 6-hydroxydopamine-lesioned and control hemiforebrains.

One week after the intrastriatal injection of 6-hydroxydopamine, mice were injected with apomorphine (0.25 mg/kg, s.c.). Those mice with ten or more net contralateral turns/2 minutes were used in this experiment. Hydrazinomethyldopa (HMD, 50 mg/kg) was injected subcutaneously one hour before administration of L-dopa (100 or 200 mg/kg i.p.). The mice were sacrificed one hour after the injection of L-dopa and two hemiforebrains taken for simultaneous analysis of dopa, dopamine and 5-hydroxytryptamine.

Open bars indicate the nonlesioned hemisphere and the solid bars represent the 6-hydroxydopamine-lesioned hemisphere. The height of each bar represents the mean and vertical lines are one standard error as determined from 10 mice. The only statistically significant difference ($p<.05$) between the lesioned and control hemiforebrain was the decreased dopamine content on the lesioned side.

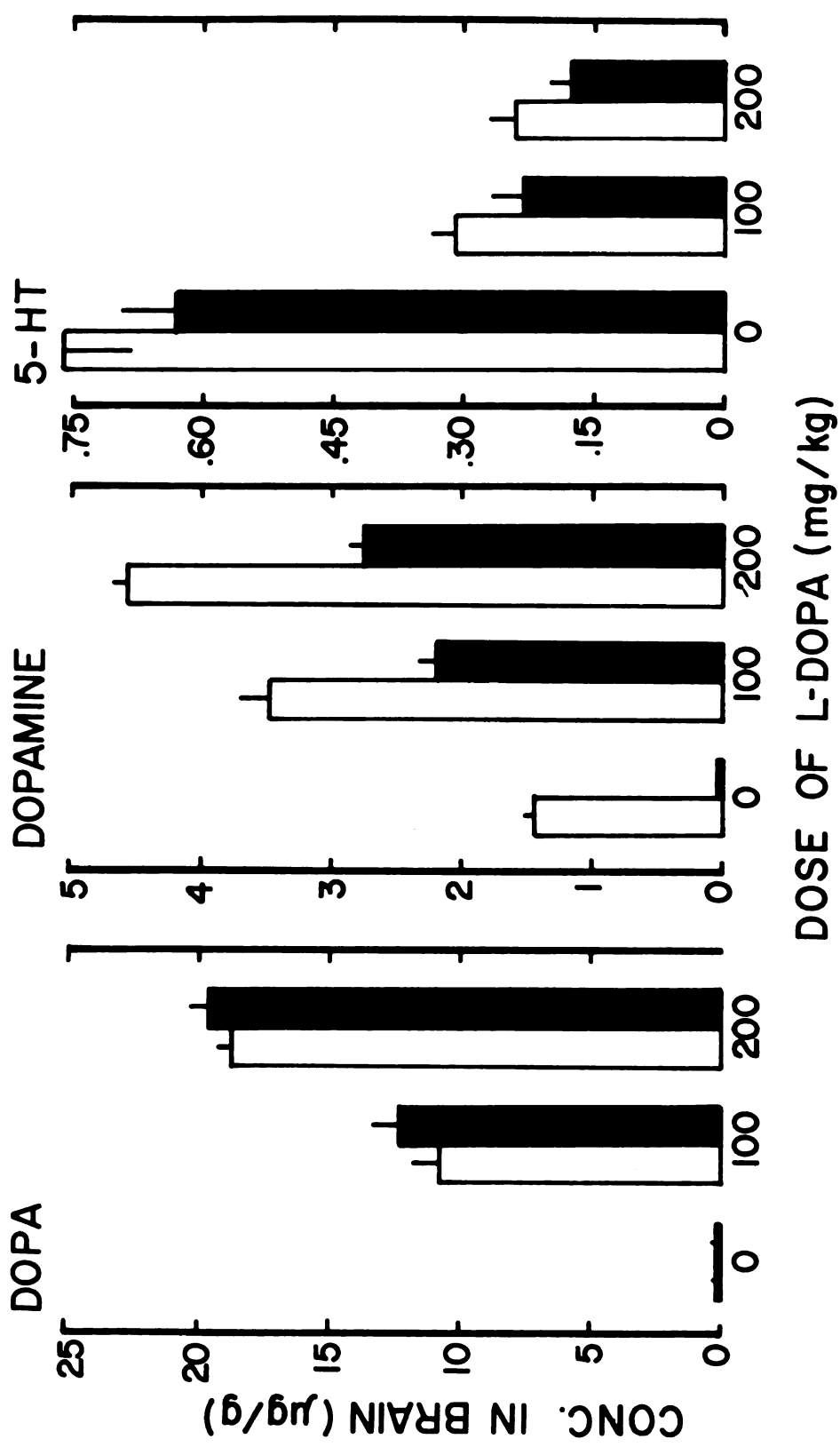

Figure 14. Comparison of L-dopa-induced depletion of 5-hydroxytryptamine in 6-hydroxydopamine-lesioned and control hemiforebrains.

Figure 15. Comparison of high doses of L- and D-dopa on 5-hydroxytryptamine (5-HT) depletion in mouse forebrain.

Control mice were injected with hydrazinomethyldopa (HMD, 50 mg/kg s.c.) one hour before the injection of methylcellulose vehicle (solid bars) L-dopa (0.1 or 0.2 g/kg; lined bars) or D-dopa (1 or 2 g/kg; open bars). The mice were sacrificed one hour after dopa administration and the brain removed. Two hemiforebrain were pooled for the simultaneous analysis of dopa, dopamine and 5-hydroxytryptamine.

The height of the bars represents the mean of ten experiments and the vertical line represents one standard error. All of the biochemical changes observed were significant ($p<.05$) except for the 5-HT concentration following the low dose of D-dopa.

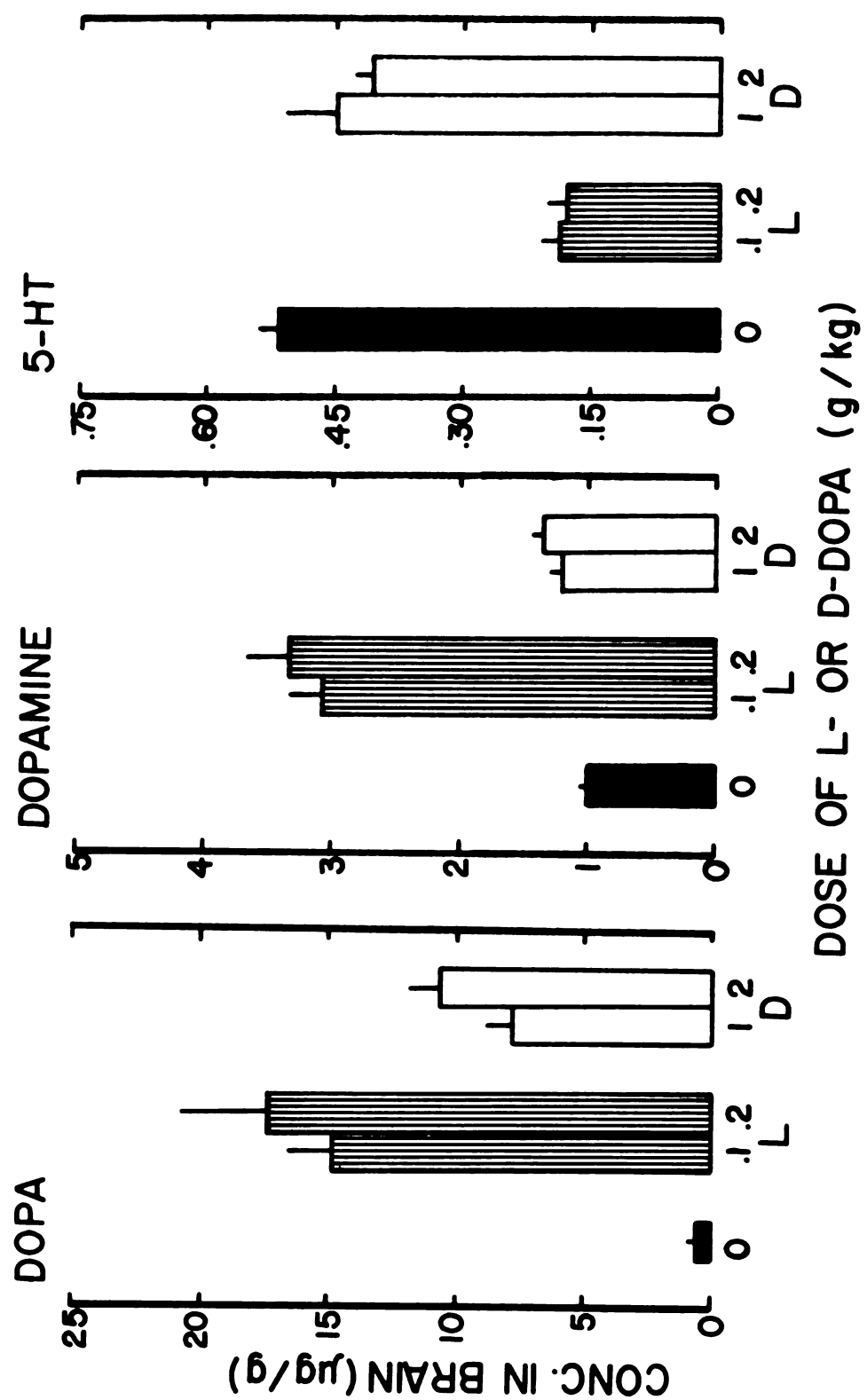

Figure 15. Comparison of high doses of L- and D-dopa on 5-hydroxytryptamine (5-HT) depletion in mouse forebrain.

forebrain concentrations of dopa and dopamine with a simultaneous reduction in the concentration of 5-HT. Administration of ten fold higher dose of D-dopa resulted in a smaller increase in forebrain dopa content than was seen with L-dopa (figure 15). Also, the increase in forebrain dopamine following D-dopa, although small, is statistically significant. The forebrain content of 5-HT was only marginally reduced by D-dopa, the effect of only the highest dose being statistically significant.

4. Turnover of 5-hydroxytryptamine in mice with unilateral 6-hydroxydopamine lesions of the striatum

Previous studies by Blondaux et al. (1973), Hery et al. (1973) and Peters et al. (1974) have suggested that 6-hydroxydopamine-induced degeneration of catecholamine neurons altered the rate of 5-HT synthesis in the forebrain. In an effort to examine further the hypothesis of an interneuronal regulation of 5-HT synthesis by catecholamine neurons, the turnover rate of 5-HT was studied in mice with intrastriatal 6-hydroxydopamine lesions.

The accumulation of 5-HT following monoamine oxidase inhibition by pargyline is presented in figure 16 for control and hemiforebrains with intrastriatal 6-hydroxydopamine lesions. A linear increase in the endogenous 5-HT concentration in both the lesioned and nonlesioned hemiforebrains was demonstrated by correlation coefficients of r=.596 (nonlesioned) and r=.415 (lesioned) as determined by the least squares linear regressional analysis. The 95% confidence intervals for the slopes were analyzed (Goldstein, 1964) and found

Figure 16. 5-Hydroxytryptamine (5-HT) turnover in 6-hydroxydopamine-lesioned and control hemiforebrains.

One week following the intrastriatal injection of 6-hydroxydopamine, mice were tested with apomorphine and those mice with ten or more turns/2 minutes were used in this study. Pargyline (150 mg/kg) was injected intraperitoneally 0, 10 and 20 minutes before sacrifice. The forebrain was removed and 2 hemiforebrains pooled for the organic extraction procedure.

The mean concentration of 5-Hydroxytryptamine in the lesioned (●) and in the nonlesioned hemiforebrain (O) is represented at each time point with a vertical line indicating one standard error as determined in eight animals. The solid line for the nonlesioned side and the dashed line for the lesioned side represent the regression lines as calculated by the method of least squares.

*Correlation is statistically significant.

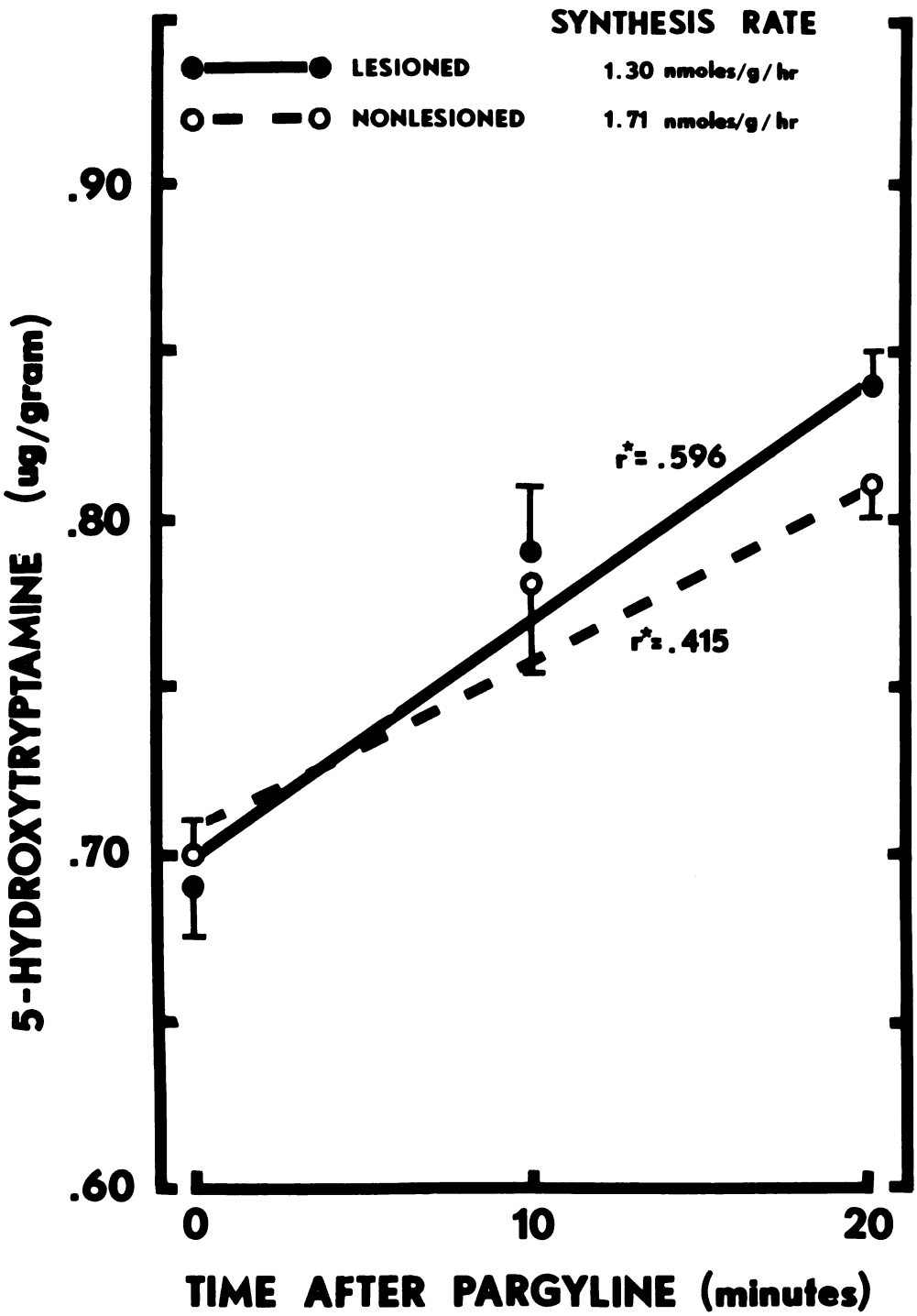

Figure 16. 5-Hydroxytryptamine turnover in 6-hydroxydopamine-lesioned and control hemiforebrains.

to be different than zero. Furthermore, there was no statistical difference between the slopes of the lesioned versus the nonlesioned side when compared using the Student's "t" test ($p<0.01$). Thus, the 5-HT synthesis rates as determined from the slopes of the lines were not significantly different. The synthesis rates of 1.71 nmoles/gram/hour (nonlesioned hemiforebrain) and 1.30 nmoles/gram/hour (lesioned hemiforebrain) were found to be in good agreement with Tozer et al. (1966) and Lin et al. (1969b), but slightly lower than those reported by Morot-Gaudry et al. (1974).

An additional experiment was performed to determine if the synthesis rate of 5-HT neurons might be altered by the administration of L-dopa. Figure 17 is a comparison of the 5-HT synthesis rates in the lesioned and nonlesioned hemisphere after the administration of L-dopa (20 mg/kg). Following the injection of pargyline there was a linear increase in the endogenous 5-HT concentrations. The correlation coefficients as determined by least squares regressional analysis were $r=.304$ (nonlesioned hemisphere) and $r=.411$ (lesioned hemisphere). The slopes of the lines for the lesioned and nonlesioned hemispheres were found to be different from zero but not significantly different from each other ($p<0.05$). The 5-HT synthesis rates of 1.87 nmoles/gram/hour (nonlesioned hemisphere) and 2.09 nmoles/gram/hour (lesioned hemisphere) for the L-dopa treated mice were not significantly different from the synthesis rates in mice which did not receive L-dopa (figure 16) as determined by a comparison of the slopes of the lines ($p<.05$).

Figure 17. 5-Hydroxytryptamine (5-HT) turnover in 6-hydroxydopamine-lesioned and control hemiforebrains following the administration of L-dopa.

One week after the intrastriatal injection of 6-hydroxydopamine, mice were tested with apomorphine (0.25 mg/kg s.c.). Those mice with ten or more contralateral turns/2 minutes were used in this study. Pargyline (150 mg/kg) was injected intraperitoneally 0, 10 and 20 minutes before sacrifice. L-dopa (20 mg/kg) was injected 15 minutes before pargyline.

The mean concentration of 5-hydroxytryptamine in the lesioned (●) and nonlesioned (○) hemiforebrain is represented at each time point with a vertical line indicating one standard error as determined in six animals. The solid line for the nonlesioned side and the dashed line for the lesioned side represent the regression lines as calculated by the method of least squares.

*Correlation is statistically significant.

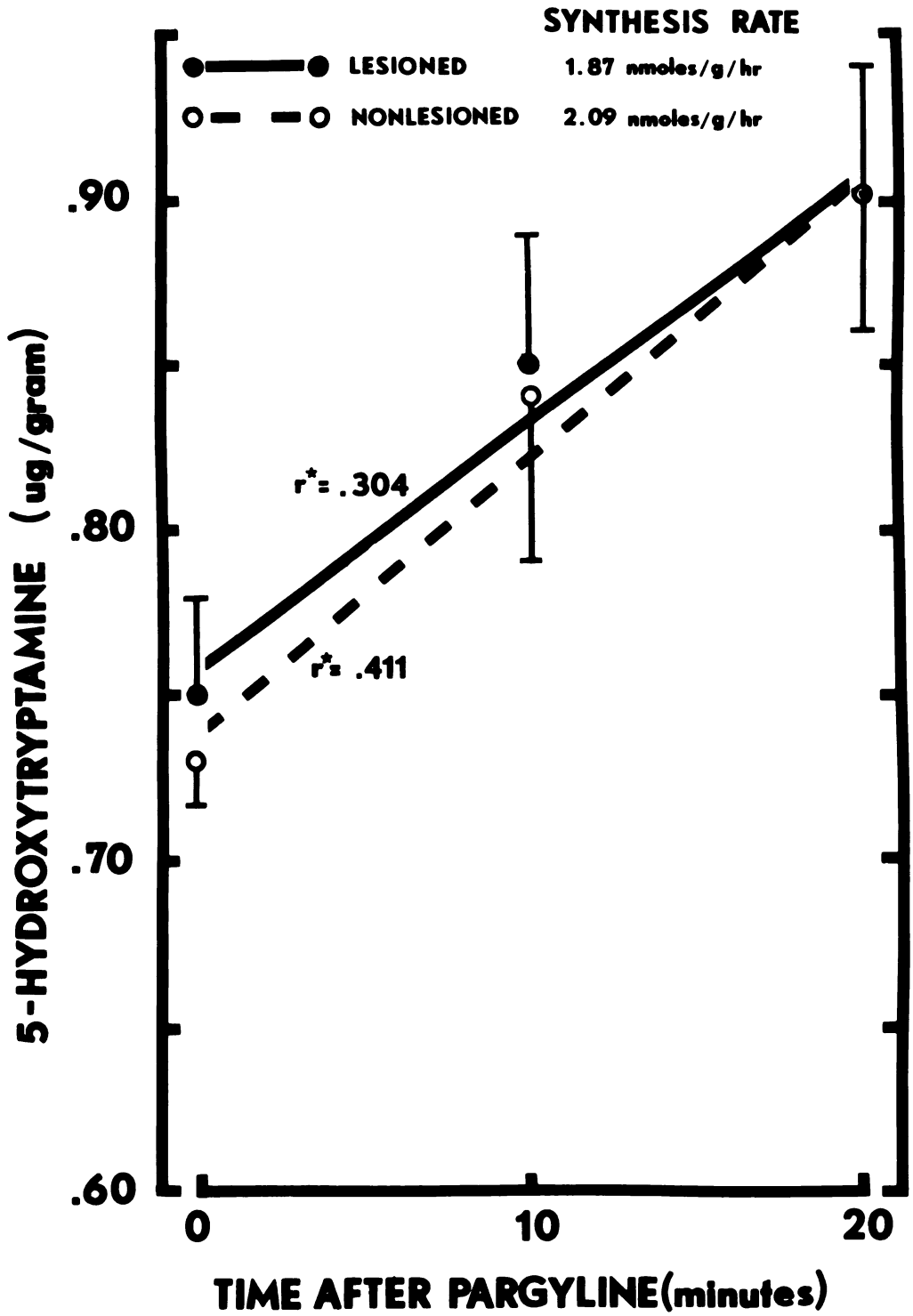

Figure 17. 5-Hydroxytryptamine (5-HT) turnover rate in 6-hydroxydopamine-lesioned and control hemiforebrains following the administration of L-dopa.

5. Utility of 5,7-dihydroxytryptamine for producing selective depletion of brain 5-HT

As discussed previously, 5,7-dihydroxytryptamine has been proposed to represent an improvement over 5,6-dihydroxytryptamine in the chemical lesioning of serotonergic neurons (Baumgarten and Lachenmayer, 1972; Daly et al., 1974; Gershon and Baldessarini, 1974).

Thus, a pilot study was initiated to determine the utility of 5,7-dihydroxytryptamine intracerebral injections for producing selective depletion of brain 5-HT.

A comparison of different doses of 5,7-dihydroxytryptamine (10-100 μg) on the hemiforebrain concentration of monoamines is presented in Table 4. Although the data represent the mean of only two experiments, the injection of a low dose of 5,7-dihydroxytryptamine (10 μg) into the left striatum caused a reduction of 5-HT and 5-HIAA to approximately 67 and 59% of nonlesioned mice. At higher doses of 5,7-dihydroxytryptamine (25, 50 and 100 μg) 5-HT and 5-HIAA were reduced on the lesioned side but the 5-HT content of the nonlesioned hemiforebrain was also reduced. In other experiments, the injection of 5,7-dihydroxytryptamine in a volume of 2 μl also decreased 5-HT and 5-HIAA bilaterally.

The reduction of 5-HT and 5-HIAA observed in Table 4 has been demonstrated previously by Jacoby et al. (1974). The nonspecificity of this chemical lesioning technique was apparent by the reduction of dopamine content at the 10 μg dose and norepinephrine at the 25 μg dose (Table 4). The higher doses of 5,7-dihydroxytryptamine resulted in a further reduction of dopamine on the

Table 4. Effect of left intrastriatal injection of 5,7-dihydroxytryptamine on forebrain monoamine concentrations.

Treatment	Hemisection	HEMIFOREBRAIN MONOAMINE CONCENTRATIONS PERCENT OF CONTROL			
		5-HT	5HIAA	DOPAMINE	NOREPINEPHRINE
10 µg 5,7-DHT	left	67.0± 6.6	59.4±10.85	81.6± 3.7	100.6±3.9
	right	74.6± 1.7	79.1± 0.00	107.5± 7.5	100.0±4.2
25 µg 5,7-DHT	left	58.5± 1.9	47.9± 1.05	72.1± 1.0	67.4±2.2
	right	54.3±11.9	79.1± 2.35	88.6± 0.5	77.1±2.1
50 µg 5,7-DHT	left	51.4±16.3	56.2± 8.8	39.0± 2.9	46.2±7.7
	right	59.1± 4.5	54.7± 2.4	117.3±24.4	55.2±0.0
100 µg 5,7-DHT	left	41.9± 6.8	66.7±15.8	39.6±10.5	44.3±2.0
	right	44.0± 4.6	31.4± 0.0	122.1± 4.2	44.8±6.9

Ten days following the injection of 5,7-dihydroxytryptamine (5,7-DHT) into the left striatum, mice were sacrificed and two left or two right hemiforebrains were pooled for biochemical analyses. The injection vehicle was 4 microliters of distilled water containing 0.8 µg sodium ascorbate. The biochemical analyses were performed following organic extraction of the brain tissue. The values presented are expressed as percent of control noninjected mice and represent the mean of two experiments ± one standard error. 5-HT = 5-hydroxytryptamine; 5HIAA = 5-hydroxyindoleacetic acid.

lesioned side and of norepinephrone on both the lesioned and non-lesioned side. Thus, 5,7-dihydroxytryptamine was not useful in selectively lesioning serotonergic neurons and therefore was not utilized in further studies.

DISCUSSION

The principal objective of this dissertation was to examine in depth the theory that L-dopa exerts its behavioral and biochemical effects through a prerequisite conversion to dopamine. At the inception of this thesis the idea that L-dopa *per se* might have some dopaminergic receptor stimulating properties was considered to be a tenable hypothesis. The clinical literature was devoid of any reports that inhibition of cerebral AAAD activity blocked the ameliorative effects of L-dopa in the treatment of parkinsonism. In fact, a few clinicians have suggested that peripheral and central AAAD inhibition by NSD 1055 (Howse and Matthews, 1973) or α-methyldopa (Fermaglich and O'Doherty, 1971a,b; Sweet *et al.*, 1972) may have potentiated the therapeutic action of L-dopa. Thus, there was not a clear delineation of the role that dopaminergic receptor stimulation in the various behavioral and neurochemical effects of L-dopa.

A. D-Dopa-induced contralateral circling

L-Dopa induces contralateral circling in unilateral 6-hydroxydopamine-lesioned rodents presumably because of a conversion to dopamine which then stimulates supersensitive postsynaptic receptors (Ungerstedt, 1971; Von Voigtlander and Moore, 1973). The unexpected finding of a dose-related increase in contralateral circling following the administration of D-dopa (figure 9B) initiated a reconsideration of the previous assumption.

There were many reasons why the D-dopa-induced contralateral circling brought about a reconsideration of the importance of dopamine formation in the mechanism of L-dopa action. First, D-dopa was not considered a substrate for cerebral AAAD, as determined by incubating purified AAAD with ^{14}C-D-dopa (Sims et al., 1973), and therefore would not have been expected to be decarboxylated to dopamine. Metabolism of D-dopa to dopamine by other metabolic routes was not considered likely since the mouse forebrain had been shown not to contain any detectable D-amino acid oxidase activity (Goldstein, 1966). Furthermore, investigators had not been able to detect the cerebral formation of dopamine following the administration of either ^{14}C-D-dopa (Shindo and Maeda, 1973) or D-dopa at 500 mg/kg (Weiss et al., 1974).

Thus, D-dopa-induced contralateral circling was considered to be potentially indicative of direct dopaminergic receptor activation (figure 4, step 10), since the previous studies had provided evidence against conversion of D-dopa to dopamine in the brain. Even though receptors to certain drugs have been shown to be stereoselective (Patil et al., 1974), it was considered theoretically possible that at least a portion of the response to L-dopa may also be attributable to a direct dopaminergic receptor activation (figure 4, step 9).

B. Enantiomeric purity of D- and L-dopa

Approximately ten times as much D-dopa had to be administered to produce contralateral circling rates equivalent to those produced by L-dopa (i.e., 10 mg/kg of L-dopa, figure 9A and 100 mg/kg of D-dopa, figure 9B). The major criticism raised at this point was that the response to D-dopa may be due to an impurity of L-dopa in our sample. Thus, the administration of D-dopa containing a small contaminant of L-dopa could induce the contralateral circling shown in figure 9B as the direct result of decarboxylation of the L-isomer.

The specific rotation values of our D-dopa samples were approximately equivalent to those reported in the literature (Table 2). Thus, the D-dopa samples taken from different sources did not appear to contain any L-isomer as contaminant. If the circling response to D-dopa was due to a contaminant of L-dopa then the D-dopa would have to contain approximately 10% of the L-isomer as an impurity (compare figures 9A and 9B). D-Dopa samples were prepared containing 10% L-dopa (w/w) and analyzed for their optical activity. The values for specific rotation changed from +12.00, +12.55 and +11.95 for the samples containing 100% D-dopa to +9.95 and +9.95 for the samples containing 90% D-dopa and 10% L-dopa. Thus, the D-dopa samples were considered not to contain L-dopa as an impurity in sufficient quantities to account for the D-dopa-induced contralateral circling.

C. Endogenous brain dopamine concentrations following administration of L- or D-dopa

The enzyme, AAAD (figure 4, step 2), is not saturated with substrate and therefore the administration of L-dopa to rodents results in an increase in cerebral dopamine content (Thut and Rech, 1972; Horst et al., 1973; Weiss et al., 1974). D-Dopa, however, should not elevate cerebral dopamine concentrations since the enzymes necessary for its metabolism are not present in the brain as was previously discussed.

After the intraperitoneal administration of L- and D-dopa at doses which produced a similar number of contralateral turns/2 minutes (10 mg/kg and 100 mg/kg, respectively; figure 9), the increases in forebrain dopa concentrations were approximately equivalent (figure 13). Following the administration of L-dopa (10 mg/kg) to mice pretreated with HMD (25 mg/kg), an increase in forebrain dopamine concentration was observed (figure 13) as demonstrated previously (Thut and Rech, 1972; Horst et al., 1973). However, following the administration of D-dopa (100 mg/kg) to mice pretreated with HMD (25 mg/kg) no significant increase in forebrain dopamine concentration was observed (figure 13), confirming the previous report by Weiss and co-workers (1974).

Thus, after the administration of L- and D-dopa at doses which produce a similar number of contralateral turns/2 minutes (figure 9), the increases in forebrain dopa concentration appeared better correlated with the contralateral circling behavior than did

changes in forebrain dopamine concentrations (figure 13). It was proposed, therefore, that L- or D-dopa may possibly have some dopaminergic receptor stimulating properties (Goodale and Moore, 1975).

D. Peripheral decarboxylase inhibition and dopa-induced contralateral circling

Hydrazinomethyldopa (HMD) at doses of 10 to 500 mg/kg has been shown to enhance the increase in cerebral ^{14}C-catecholamines seen following administration of ^{14}C-dopa (Bartholini and Pletscher, 1969). Further evidence of a selective inhibition of peripheral AAAD activity is presented in Table 3. The HMD pretreatment times of 60 or 225 minutes (table 3) were selected such that a comparison could be made between the contralateral circling in figure 12A and the forebrain concentrations of ^{3}H-dopa and ^{3}H-dopamine at selected time intervals. Thus, the administration of HMD at 60 minutes before intravenous ^{3}H-L-dopa measures the cerebral conversion of ^{3}H-dopa to ^{3}H-dopamine corresponding to the time interval between 0 and 15 minutes on the abscissa of figure 12A. The administration of HMD at 225 minutes before intravenous ^{3}H-L-dopa measures the cerebral conversion of ^{3}H-dopa to ^{3}H-dopamine corresponding to the time interval between 165 and 180 minutes on the abscissa of figure 12A.

As presented in Table 3 pretreatment with HMD at 60 or 225 minutes before intravenous ^{3}H-L-dopa resulted in increases of cerebral ^{3}H-dopa and ^{3}H-dopamine concentrations, demonstrating a selective inhibition of peripheral AAAD activity.

Pretreatment of 6-hydroxydopamine-lesioned mice with HMD resulted in an enhanced and prolonged contralateral circling response to L-dopa (figure 12A). The duration of L-dopa-induced contralateral circling was prolonged from 45 minutes with vehicle pretreatment to 180 minutes with HMD pretreatment. The results presented in figure 12A have been substantiated by Mendez et al. (1975) who demonstrated that HMD (4 mg/kg) prolonged the L-dopa-induced contralateral circling responses in rats.

Comparison of ^{3}H-dopa and ^{3}H-dopamine concentrations following HMD pretreatment (table 3) to the circling behavior in figure 12A does not permit any discrimination between the role of dopa or dopamine in mediating the L-dopa-induced contralateral circling. Thus, when the L-dopa-induced contralateral circling was observed at 15 and 180 minutes (figure 12A) both ^{3}H-dopa and ^{3}H-dopamine concentrations were elevated. It is important to emphasize that ^{3}H-dopamine concentrations at 225 minutes (table 3, HMD) represent the relative amount of cerebral dopamine formed from ^{3}H-L-dopa administered 15 minutes previously and not the amount of dopamine which would be present following the administration of L-dopa 180 minutes previously.

The enhanced circling response to L-dopa by HMD was expected, but HMD also prolonged and enhanced the D-dopa-induced contralateral circling. The question must now be asked: how can a drug which blocks the decarboxylation of L-amino acids enhance the actions of D-dopa? Partial explanation for this paradoxical

question may be obtained by studying the metabolic pathway for D-dopa in the kidney as proposed by Shindo and Maeda (figure 5). They presented evidence which suggested that D-dopa is oxidized in the rat kidney by D-amino acid oxidase to 3,4-dihydroxyphenyl-pyruvate which in turn is transaminated to L-dopa. The L-dopa is then rapidly converted by AAAD in the kidney to dopamine and this amine is excreted in the urine. If the last decarboxylation step is blocked by HMD, the L-dopa which is synthesized from D-dopa in the kidney may be reabsorbed into the systemic circulation and transported to the brain. There it can be decarboxylated to dopamine which stimulates the supersensitive receptors in the striatum. This represents one theoretical mechanism by which HMD prolongs and enhances the contralateral circling responses to D-dopa.

Previous investigators have utilized NSD 1055 (brocresine) as an AAAD inhibitor *in vitro* (Levine and Sjoerdsma, 1964; Ellenbogen *et al.*, 1973; Miller *et al.*, 1974b) and as an inhibitor of peripheral and cerebral AAAD activity *in vivo* (Levine and Sjoerdsma, 1964; Robson, 1971; Roberge *et al.*, 1972; Howse and Matthews, 1973). Studies were initiated to determine the effect of NSD 1055 on cerebral AAAD activity and on the L-dopa-induced contralateral circling. Pretreatment with NSD 1055 30 minutes before intravenous administration of ^{3}H-L-dopa resulted in increased cerebral concentrations of ^{3}H-dopa and ^{3}H-dopamine (Table 3). Thus, the increased concentrations of ^{3}H-dopa indicate an inhibition of peripheral AAAD

activity while the increased concentrations of ^{3}H-dopamine indicate that NSD 1055 does not appreciably inhibit cerebral AAAD activity. The results presented in Table 3 confirm the studies by Lazare and Watson (1972) showing that NSD 1055 does not appreciably inhibit cerebral AAAD activity.

Pretreatment of 6-hydroxydopamine-lesioned mice with NSD 1055 prolonged the contralateral circling responses induced by L- or D-dopa (Table 1). At fifteen minutes, when the L-dopa-induced contralateral circling following HMD pretreatment was increased, there was an increase in both ^{3}H-dopa and ^{3}H-dopamine concentrations (Table 3, NSD 1055). Thus, comparison of Tables 1 and 3 does not permit differentiation between the role of dopa and dopamine in mediating the L-dopa-induced contralateral circling. Prolongation of the D-dopa-induced contralateral circling by NSD 1055 may be attributable to inhibition of renal AAAD activity as previously discussed for HMD.

α-Methyldopa has been utilized to inhibit peripheral and central AAAD activity *in vivo* (Murphy and Sourkes, 1961; Levine and Sjoerdsma, 1964; Vazquez and Sabelli, 1975) and has also been used to inhibit AAAD activity during *in vitro* studies (Levine and Sjoerdsma, 1964; Firnau *et al.*, 1976). The administration of α-methyldopa 30 minutes before ^{3}H-L-dopa resulted in increased forebrain concentrations of both ^{3}H-dopa and ^{3}H-dopamine (Table 3). These results confirm the findings of Bartholini and Pletscher (1969) showing that α-methyldopa is not an effective inhibitor of

cerebral AAAD activity even at high doses. The effect of α-methyldopa pretreatment on the L- or D-dopa-induced contralateral circling response was not studied.

E. Central decarboxylase inhibition and dopa-induced contralateral circling

The initial studies by Burkard et al., (1962, 1964), Pletscher and Gey (1963), Bartholini et al. (1967, 1969) and Bartholini and Pletscher (1969), thoroughly characterizing the inhibition of cerebral AAAD activity by Ro44602, have resulted in many investigators utilizing this compound to study the mechanism of L-dopa action in the central nervous system (Lotti and Porter, 1970; Ungerstedt, 1971; Garelis and Neff, 1973; Bunney et al., 1973; Reichenberg and Vetulani, 1973; Thut, 1974). Further evidence for the inhibition of cerebral AAAD activity by Ro44602 is presented in Table 3. The Ro44602 pretreatment times of 30 or 195 minutes (Table 3) were selected such that a comparison could be made between the contralateral circling in figure 10A and the forebrain concentration of ^{3}H-dopa and ^{3}H-dopamine at selected time intervals. Thus, the administration of Ro44602 at 30 minutes before intravenous ^{3}H-L-dopa measured the cerebral conversion of ^{3}H-dopa to ^{3}H-dopamine corresponding to the time interval between 0 and 15 minutes on the abscissa of figure 10A. The administration of Ro44602 at 195 minutes before intravenous ^{3}H-L-dopa measured the cerebral conversion of ^{3}H-dopa to ^{3}H-dopamine corresponding to the time interval between 165 and 180 minutes on the abscissa of figure 10A.

Pretreatment of mice with Ro44602 at 30 or 195 minutes prior to ^{3}H-dopa caused a large increase in the forebrain ^{3}H-dopa concentration, reflecting an inhibition of peripheral AAAD. Furthermore, the forebrain concentrations of ^{3}H-dopamine were reduced at 30 minutes, but increased at 195 minutes after Ro44602 administration. A reduction in the cerebral concentration of Ro44602, below that which is necessary to appreciably inhibit cerebral AAAD activity, would account for the increased ^{3}H-dopamine concentrations at 195 minutes.

Previous studies by Ungerstedt (1971b) have demonstrated that with increasing doses of Ro44602 there was a corresponding increase in the delay in onset of the L-dopa-induced contralateral circling. As demonstrated in figure 10A, pretreatment of 6-hydroxydopamine-lesioned mice with Ro44602 produced an initial delay followed by a prolonged period of circling in response to the administration of L-dopa.

Comparison of the ^{3}H-dopamine concentrations at various times after Ro44602 pretreatment to the circling behavior in figure 12A suggests a direct correlation between the forebrain concentrations of ^{3}H-dopamine and the contralateral circling response to L-dopa. Following Ro44602 administration, the L-dopa-induced contralateral circling response is blocked at 15 minutes and the cerebral increase in ^{3}H-dopamine concentration is also reduced at the corresponding time interval of 30 minutes (Table 3). Furthermore, the L-dopa-induced contralateral circling is increased at 180 minutes

as is the forebrain ^{3}H-dopamine concentration. However, comparison of the circling behavior and the ^{3}H-dopa concentrations at various times after Ro44602 suggests that there is no direct correlation between the ^{3}H-dopa concentration with the contralateral circling response. Thus, the L-dopa-induced contralateral circling is blocked at 15 minutes and elevated at 180 minutes (figure 10A) while the forebrain ^{3}H-dopa concentrations are elevated at both corresponding time points of 30 and 195 minutes (Table 3).

As demonstrated in figure 10B when D-dopa was administered following pretreatment with Ro44602, the contralateral circling was blocked for 60 minutes. At 90 minutes an increase in contralateral circling occurred and was still elevated 360 minutes after D-dopa administration. Thus, these behavioral studies with Ro44602 suggest that the D-dopa-induced contralateral circling, by analogy to the similar circling response obtained with Ro44602 and L-dopa, may be mediated by dopamine formed in the denervated striatum. The firm conclusion that dopamine mediates the D-dopa-induced contralateral circling would be made except for the antithetical biochemical data in figure 13 which do not corroborate such a theory. If the circling response is directly correlated with increased dopamine concentrations then the threshold dose for eliciting contralateral circling (i.e., 10 mg/kg of L-dopa, figure 9A and 100 mg/kg of D-dopa, figure 9B) should induce similar increases in forebrain dopamine concentrations since this, theoretically, is the threshold concentration of dopamine in the forebrain for eliciting

contralateral circling. However, in contrast to increase in dopamine concentration (0.50±.08 μg/g) following L-dopa (10 mg/kg) there was no increase seen in the dopamine concentration (0.07±.04 μg/g) following D-dopa (100 mg/kg) administration. Thus, the administration of L- or D-dopa, at doses which induce increases in contralateral circling, produce similar increases in dopa concentration but dissimilar changes in dopamine concentration, suggesting that D-dopa-induced contralateral circling is better correlated with the increases in dopa concentration. In summary, D-dopa-induced contralateral circling is blocked by the AAAD inhibitor, Ro44602, while the administration of D-dopa produces no increases in forebrain dopamine concentrations. These paradoxical findings could be explained, if Ro44602 was initially inhibiting the contralateral circling through a pharmacological mechanism other than cerebral AAAD inhibition. Therefore, another cerebral AAAD inhibitor, NSD 1015, was studied to compare its effect on the L- and D-dopa-induced contralateral circling with the increases in ^{3}H-dopa and ^{3}H-dopamine concentrations.

NSD 1015 was initially shown to be an effective inhibitor of cerebral AAAD activity by Bartholini and Pletscher (1969). Subsequently, Carlsson and Lindqvist (1973) demonstrated that NSD 1015 at 100 mg/kg i.p. effectively inhibited cerebral AAAD activity and this inhibition, as measured by dopa accumulation, was linear for 30 minutes. Longer time intervals were not studied. Further evidence for the inhibition of cerebral AAAD activity by NSD 1015 is presented in Table 3. The administration of NSD 1015 at 30 minutes before intravenous ^{3}H-L-dopa corresponds to the time

interval between 0 and 15 minutes on the abscissa of figure 11A. The administration of NSD 1015 at 195 minutes before intravenous ^{3}H-L-dopa corresponds to the time interval between 165 and 180 minutes on the abscissa of figure 11A.

Pretreatment of mice with NSD 1015 at 30 minutes prior to intravenous ^{3}H-L-dopa caused a large increase in the forebrain concentration of ^{3}H-dopa, with a concomitant reduction of ^{3}H-dopamine concentration. Thus, at 15 minutes there was inhibition of both peripheral and central AAAD activity. Pretreatment with NSD 1015, 195 minutes before intravenous ^{3}H-L-dopa, resulted in markedly elevated ^{3}H-dopa concentration, as seen following peripheral AAAD inhibition with HMD (Table 3). However, the ^{3}H-dopamine concentrations were not significantly increased as seen when only the peripheral AAAD activity is inhibited by HMD (Table 3), thus indicating that at 195 minutes there is still an appreciable amount of residual AAAD inhibition by NSD 1015 in the brain.

Previously, NSD 1015 has not been utilized by investigators to study the behavioral actions of L-dopa. Pretreatment with NSD 1015 (100 mg/kg) completely blocked the L-dopa-induced contralateral circling as demonstrated in figure 11A. The circling did not recur at later time intervals as evidenced by -0.83±0.72 (mean ± 1 S.E.) turns/2 minutes at 180 minutes after L-dopa administration.

Since the L-dopa-induced contralateral circling (figure 11A) and the increase in cerebral ^{3}H-dopamine (Table 3) are both blocked 30 minutes after NSD 1015, the data at this time supports the

theory of a prerequisite conversion of L-dopa to dopamine as mediating the L-dopa-induced contralateral circling. The presence of residual AAAD inhibition at 195 minutes after NSD 1015, concurrent with inhibition of L-dopa-induced contralateral circling, supports a correlation between the L-dopa-induced contralateral circling and the ^{3}H-dopamine concentration in the forebrain.

As demonstrated in figure 11B, the inhibition of contralateral circling by NSD 1015 was not due to a postsynaptic action, such as receptor blockade, since NSD 1015 did not alter the contralateral circling induced by apomorphine.

Pretreatment with NSD 1015 blocked the D-dopa-induced contralateral circling presented in figure 11C. As discussed previously this presents the paradoxical situation of an AAAD inhibitor blocking the behavioral actions of D-dopa which has previously been shown not to increase dopamine concentrations (figure 13).

F. Conclusions on the dopaminergic receptor agonistic properties of L- and D-dopa

The above comparative studies on the cerebral concentrations of ^{3}H-dopa and ^{3}H-dopamine, correlated with periods of active or quiescent contralateral circling, corroborate the theory that the administration of L-dopa results in the activation of postsynaptic dopaminergic receptors following a prerequisite conversion of L-dopa to dopamine. Thus, during the periods of increased contralateral circling there were concurrent increases in the ^{3}H-dopamine concentrations. Likewise, during periods when the contralateral

circling was not observed, the ^{3}H-dopamine concentrations were reduced or not significantly different from controls.

The similarity between the responses of L- and D-dopa to cerebral AAAD inhibition would intimate that D-dopa induces activation of postsynaptic dopaminergic receptors following conversion of small amounts of D-dopa to dopamine. However, since behaviorally relevant doses of D-dopa failed to increase dopamine concentrations, the conversion of D-dopa to dopamine is not an absolute requirement for the stimulation of postsynaptic dopaminergic receptors. Furthermore, there is no evidence in the literature to suggest that D-dopa is converted to dopamine in the brain. Therefore, although conversion of D-dopa to dopamine represents a potential mechanism for activation of postsynaptic dopaminergic receptors other undefined or unappreciated biochemical transformations of D-dopa may not be excluded as important constituents of the D-dopa-induced contralateral circling response.

Since it has been proposed that L-dopa is converted to dopamine before activation of dopaminergic receptors, the question arises about how the previous clinical findings of an enhanced therapeutic effect of L-dopa with a central AAAD inhibitor are explained. From the results presented in Table 2, neither NSD 1055 (brocresine) nor α -methyldopa inhibited the conversion of L-dopa to dopamine in the brain. Thus, the potentiation of the therapeutic efficacy of L-dopa probably resulted from inhibition of the peripheral AAAD activity increasing the amount of dopa transported into the brain.

Even though direct receptor stimulation by L-dopa is not corroborated by the data presented in this thesis, it is important to emphasize that the mouse circling test is far from being an exact replication of the biochemical abnormalities found in Parkinson's disease. Receptor alterations due to species differences or disease may make direct receptor stimulation by L-dopa responsible for a significant part of the beneficial or detrimental responses seen during clinical therapy with L-dopa.

G. Depletion of 5-hydroxytryptamine by L-dopa in 6-hydroxydopamine-lesioned mice

The L-dopa-induced depletion of brain 5-HT was studied in an effort to determine whether dopamine formation and 5-HT depletion would precede independent of dopaminergic nerve terminals. Conflicting results have been reported previously concerning this question (Uretsky and Schoenfeld, 1971; Poirier et al., 1971; Ng et al., 1972; Langelier et al., 1973; Lytle et al., 1972).

The specificity of the intrastriatal 6-hydroxydopamine lesion technique is evidenced by the reduction of dopamine to 12% of control on the lesioned side while not altering the 5-HT or dopa concentrations (figure 14). The administration of L-dopa resulted in a decrease of 5-HT content in the lesioned side of the forebrain which was not significantly different from the control side. Thus, the data presented in figure 14 suggest that the L-dopa-induced depletion of 5-HT is unaltered by the absence of dopaminergic nerve terminals.

These results support the findings of Uretsky and Schoenfeld (1971), Lytle et al. (1972) and Ng et al. (1972) who found no difference between the 5-HT content in 6-hydroxydopamine-treated and control brains following the administration of L-dopa. The question now arises about how the reports by Poirier et al. (1971) and Langelier et al. (1973) of an enhanced depletion of 5-HT following 6-hydroxydopamine are explained. These investigators utilized electrocoagulative lesions in the ventrotegmental area of the brain stem to destroy the dopaminergic neurons. Since the 5-HT content on the side of the lesion prior to L-dopa was not reported, their results may be explained by nonspecific decreases in 5-HT as a result of the brain stem lesions.

The dopamine accumulation was significantly reduced on the 6-hydroxydopamine-lesioned side following the administration of both doses of L-dopa (figure 14). This difference may be the result of less AAAD present to convert L-dopa to dopamine. Alternatively, the difference may represent a decrease in the "storage" pool of dopamine and/or a decrease in the storage capacity for newly formed dopamine. Furthermore, the increase of dopamine content observed in the hemiforebrain containing degenerated dopaminergic nerve terminals probably represents dopamine derived from the decarboxylation of L-dopa in serotonergic neurons. The latter conclusion has been made since the decarboxylase enzymes in both the dopaminergic and serotonergic neurons are identical (Dairman et al., 1975) and the amount of extraneuronal decarboxylase in glial cells has been considered negligible (Hökfelt et al., 1973).

H. Comparison of 5-hydroxytryptamine depletion by D- and L-dopa

The mechanism of L-dopa-induced 5-HT depletion was considered following *in vitro* studies to be dependent upon the decarboxylation of L-dopa (Ng *et al.*, 1970, 1971, 1972a,b). One possible method of testing such an hypothesis with *in vivo* studies would be to pretreat with a central AAAD inhibitor prior to L-dopa. However, when Ro44602 (800 mg/kg, i.p.) was administered 30 minutes prior to sacrifice, a decrease in forebrain 5-HT was observed as previously reported by Gey and Pletscher (1963). An alternative approach to answer this question has been to compare the depletion of 5-HT by D- and L-dopa (figure 15). The forebrain level of dopa accumulated following injection was much smaller with the D-isomer even though a ten fold higher dose was used. Administration of L-dopa caused a marked increase in forebrain dopamine, with D-dopa producing only a small but significant increase in dopamine.

The concentration of 5-HT was greatly reduced following both doses of L-dopa while only the highest dose of D-dopa significantly decreased cerebral 5-HT content. The depletion of 5-HT, therefore, appeared to correlate better with the increase in dopamine than with the accumulation of dopa.

One additional theory which has not been excluded by these data is that L-dopa depletes forebrain 5-HT by inhibiting uptake of L-tryptophan into the brain (Fahn *et al.*, 1975). The uptake into the brain of one neutral amino acid (L-tyrosine) has been demonstrated to be inhibited by another neutral amino acid (L-tryptophan) but not by its optical isomer (D-tyrosine) (Guroff and Udenfriend,

1962). Thus, the neutral amino acid transport system is both competitive and stereoselective. Fernstrom and Wurtman (1972) further demonstrated that the brain 5-HT content was dependent on the uptake of L-tryptophan which could be competitively inhibited by other neutral amino acids. The lack of effect of D-dopa on brain 5-HT may be related to an inability to deplete forebrain L-tryptophan in addition to its inability to form dopamine.

I. 5-Hydroxytryptamine turnover in 6-hydroxydopamine-lesioned animals

The reports by Blondaux et al. (1973) and Hery et al. (1973) of 5-HT synthesis alterations being induced as a result of catecholamine interneuronal interactions were interesting because of its potential relevance to the behavioral interactions reported between dopaminergic and serotonergic neuronal systems (Grabowska and Michaluk, 1974; Neill et al., 1972; Breese et al., 1974; Mabry and Campbell, 1973; Green and Harvey, 1974; Cools, 1974; Weiner et al., 1975; Baldessarini et al., 1975). In addition, some investigators had presented evidence for biochemical interactions between these two neuronal systems such as: increased 5-HIAA concentrations following apomorphine (Grabowska et al., 1973, 1975); increased 5-HT and 5-HIAA content following α-methylparatyrosine (Stein et al., 1974); and decreased brain tryptophan hydroxylase activity following d-amphetamine (Knapp et al., 1974). On the other hand, some investigators have demonstrated no behavioral interactions between dopaminergic and serotonergic neuronal systems (Rotrosen et al., 1972; Jacobs et al., 1975).

Studies were undertaken, therefore, to determine if there was a specific interaction of dopaminergic neurons with the turnover rate of 5-HT. As demonstrated in figure 16 intrastriatal 6-hydroxydopamine injections, which reduced hemiforebrain dopamine to less than 15% of control, had no influence on the turnover rate of 5-HT in mouse forebrain. Furthermore, the administration of L-dopa (20 mg/kg) did not induce a change in 5-HT synthesis rate in either the lesioned or nonlesioned hemisphere (figure 17). The latter results agree with the report of Corrodi et al. (1972) who found no effect of piribedil, a dopaminergic agonist, on 5-HT turnover rates when administered to normal rats. These data (figures 16 and 17) suggest that nigrostriatal dopaminergic neurons do not modulate the synthesis rate of 5-HT in mouse brain. Thus, the initial studies (Blondaux et al., 1973; Hery et al., 1973; Peters et al., 1974) may have influenced 5-HT turnover rate by altering either a direct or indirect noradrenergic input to the serotonergic neurons as has been suggested to occur in the dorsal raphé nucleus (Svensson et al., 1975; Gallager and Aghajanian, 1975; Swanson and Hartman, 1975).

J. Utility of 5,7-dihydroxytryptamine for producing selective degeneration of serotonergic neurons

Following intrastriatal injection of 5,7-dihydroxytryptamine, hemiforebrain concentrations of norepinephrine, 5-HT and 5-HIAA were reduced as reported previously (Björklund, 1973; Table 4). Although it has been reported previously that intraventricular 5,7-dihydroxytryptamine has minimal effects on brain dopamine content

(Jacoby, 1974) these data show that following intracerebral injection of 5,7-dihydroxytryptamine both brain dopamine and 5-HT were diminished. These results support the findings of Saner et al. (1974) who demonstrated that injections of low doses of 5,6-dihydroxytryptamine (0.5-10 μg) into the medial forebrain bundle of rats resulted in a considerable decrease of brain 5-HT and dopamine. Thus, injections of 5,6- or 5,7-dihydroxytryptamine into brain tissue may produce different alterations in brain monoamines than observed following injection into the cerebrospinal fluid.

The bilateral decrease of norepinephrine, 5-HT and 5-HIAA but not dopamine (Table 4) may have been related to diffusion of 5,7-dihydroxytryptamine to the hypothalamus where norepinephrine, 5-HT and 5-HIAA but not dopamine are found in high concentrations. Although smaller volumes of injection (2 μl) also produced bilateral depletion of 5-HT and 5-HIAA, this latter volume has been shown to diffuse 1.5 mm in brain tissue from the site of injection (Agid et al., 1973). Furthermore, Saner et al. (1974) and Von Voigtlander (1972) made unilateral intracerebral injections of 5,6-dihydroxytryptamine and found no significant decreases in the 5-HT content on the unlesioned side. Thus, the bilateral decrease in 5-HT, 5-HIAA and norepinephrine appears to result from diffusion of 5,7-dihydroxytryptamine to the hypothalamus.

K. Prospective view into the mechanism of action of L-dopa

As substantiated by this dissertation, L-dopa exerts at least a part of its antiparkinsonian action by replenishing the diminished concentration of dopamine within the basal ganglia. However, the

variety of problems associated with the therapeutic response has demonstrated that the mechanism of action for L-dopa is not yet completely understood (Hornykiewcicz, 1975). The "on-off" effect, where a good response to L-dopa alternates with periods of akinesia and rigidity, has been attributed to the formation of tetrahydroisoquinoline alkaloids derived from minor pathways of L-dopa metabolism (Sandler et al., 1973; Turner et al., 1974; Dougan et al., 1975). Another therapeutic side effect, L-dopa-induced dyskinesias, have remained unabated except by drug therapy with produces a concomitant increase in parkinsonian symptoms (Tarsy et al., 1975).

Another enigmatic facet of L-dopa therapy is the recently demonstrated therapeutic potentiation by melanocyte-stimulating hormone-release inhibiting factor (M.I.F.) (Woods and Chase, 1973; Fischer et al., 1974; Chase et al., 1974). Furthermore, animal studies have not elucidated a site of action where M.I.F. might potentiate the L-dopa therapeutic response, emphasizing the need for continued research into the mechanism of interaction between L-dopa and M.I.F. (Barbeau, 1975).

The parkinsonian-like side effect of neuroleptic drugs was originally considered to be directly related to blockade of CNS dopamine receptors. However, Miller and Hiley (1974) and Snyder et al. (1974) have demonstrated that in addition to dopamine receptor blockade, drugs with very potent antimuscarinic properties induce fewer extrapyramidal side effects. The additional anticholinergic property of neuroleptic drugs tends to counterbalance the anti-dopamine effect of the drug and result in no parkinsonian-like side

effects (Iversen, 1975). Thus, the preconceived notion of dopamine receptor blockade by neuroleptic drugs represented a vague understanding of a complex interaction.

An additional therapeutic measure which bears no understandable relationship to nigrostriatal function is the use of stereotaxic surgery in parkinsonism (Selby, 1971a,b). Successful lesions of the medial segment of the globus pallidus alleviate the symptoms of both rigidity and tremor. The rigidity is also effectively controlled by L-dopa but supposedly through a completely different neurological system (i.e., the dopaminergic nigrostriatal pathway).

Biochemical evidence has been reported also suggesting that levodopa may exert some of its clinical actions through different neuronal systems. Lloyd and Hornykiewicz (1973) demonstrated that with L-dopa therapy for more than one year, there was an increase in striatal glutamic acid decarboxylase activity which is abnormally low in Parkinson's disease. L-Dopa has also been shown to have other nondopaminergic effects in the brain such as: depression of S-adenosylmethionine (Wurtman et al., 1970); depletion of serotonin (Everett and Borcherding, 1968); increased acetylcholine concentration (Sethy and Van Woert, 1974; McGeer et al., 1975); decreased rate of sulfate conjugation (Rutledge and Hoehn, 1973); enhanced turnover of norepinephrine (Keller et al., 1974); decreased acetylcholine turnover (Trabucchi et al., 1975); and release of octopamine (James and Fischer, 1975).

Thus, from the myriad of side effects and biochemical interactions it seems a grossly simplistic notion that the only significant

action of L-dopa in Parkinson's disease is to replenish the diminished dopamine content of the corpus striatum. The prospect for future research, therefore, involves elucidation of the interaction between L-dopa and numerous other compounds, some with known pharmacological properties (norepinephrine) and others with as yet little known properties (tetrahydroisoquinolines). Understanding the nondopaminergic interactions of L-dopa will be instrumental in enhancing and prolonging the therapeutic response while reducing the numerous side effects.

Significant improvement of the therapeutic regime has already been accomplished by combining an extracerebral decarboxylase inhibitor with L-dopa as a single drug available for clinical use (Korten et al., 1975). Further improvements will occur as basic research provides new and innovative approaches to L-dopa therapy such as combination with the selective MAO-B inhibitor, deprenil (Birkmayer et al., 1975) or modification with as yet unknown therapeutic agents. The treatment of Parkinson's disease with L-dopa, thus serves as a classic example of drug therapy which was initiated and is continuing to be improved through research into the mechanisms of levodopa action in the central nervous system.

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