Effects of parathion on steady-state levels and turnover of norepinephrine and dopamine in selected regions of rat brain

by

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LIST OF ABBREVIATIONS

ACh	acetylcholine
AChE	acetylcholinesterase
αMPT	α-methyl-para-tyrosine
anti-ChE	anticholinesterase
AMN	atropine methylnitrate
b	slope
BS	brain stem
BuChE	butyrocholinesterase
CA	catecholamine
[CA]	concentration of catecholamine
cfg.	centrifuge
ChE	cholinesterase
CNS	central nervous system
COMT	catecholamine-O-methyltransferase
CPZ	chlorpromazine
CS	corpus striatum
CTX	cortex (cerebral)
D1	Design 1
D2	Design 2
DA	dopamine
[DA]	concentration of dopamine
d.d.H_2O	double-distilled H_2O
DFP	diisopropyl fluorophosphonate
EDTA	ethylenediaminetetraacetic acid

EEG	electroencephalogram
HC-3	hemicholinium-3
HT	hypothalamus
HVA	homovanillic acid
inh.	inhibition
i.p.	intraperitoneal
k	rate constant
K	turnover rate
LD50	median lethal dose
MAO	monoamine oxidase
MHPG	3-methoxy-4-hydroxyphenyleneglycol
NE	norepinephrine
[NE]	concentration of norepinephrine
PrChE	propionocholinesterase
SAL	spontaneous acid liberation
SPF	spectrophotofluorometer
SSL	steady-state level
tt	turnover time

INTRODUCTION

An interdependence between the cholinergic and catecholaminergic systems in the mammalian brain is proposed as a working hypothesis. Numerous findings support this proposition. Indeed, many pharmacological agents that affect the cholinergic system in the mammalian central nervous system have been shown to affect brain catecholamines (norepinephrine and dopamine) as well (cf. Literature Review).

Two parameters (steady-state levels and turnover) of brain catecholamines have been extensively studied. Cholinolytic and cholinomimetic drugs were found to have minimal effects on the steady-state levels of brain catecholamines but marked effects on their turnover. However, these experiments utilized short-acting cholinomimetics. These drugs elicit their effects during a brief time interval, which may be insufficient to adequately influence the catecholamine turnover. Also, whole brain, rather than specific regions, was analyzed for catecholamine turnover. This approach could mask effects on catecholamine turnover that would be observed in regions rich in catecholaminergic neurons. For these reasons, the observed effects of cholinomimetics on catecholamine turnover in mammalian brain have been equivocal. A study was needed which would determine the effects of a long-acting cholinomimetic on the turnover of catecholamines in specific regions of mammalian brain.

In the present study, parathion (a so-called "irreversible" or long-acting cholinomimetic) was used to enhance the endogenous cholinergic stimulation in rat brain. The effects of parathion on the steady-state levels and turnover of norepinephrine and dopamine were determined in selected

regions of rat brain. Preliminary experiments were needed to determine an appropriate experimental design to assess treatment effects on norepinephrine and dopamine turnover in the brain.

LITERATURE REVIEW

Dopamine (DA) and norepinephrine (NE) are two catecholamines (CA's) that are considered to be putative neurotransmitters in the mammalian central nervous system (CNS). Evidence to support the neurotransmitter role of NE and DA in the mammalian CNS, as well as extensive information on the general subject of CA's, has been published (Himwich and Himwich, 1964; Bloom and Giarman, 1968; Andén et al., 1969a; Blaschko and Muscholl, 1972; Baldessarini and Karobath, 1973).

Specific areas of CA research have been adequately reviewed as well (see Appendix A), therefore, a detailed description of these areas of research will not be given in this review. However, some of the findings that suggest the neurotransmitter role of NE and DA in the mammalian CNS will receive a brief discussion.

NE was first detected in mammalian brain by von Euler (1946), who determined "adrenaline equivalents" of an extract of calf brain by measuring the pressor action of the extract on cat blood pressure. Moreover, Vogt (1954) found that the concentration of NE in dog brain was unevenly distributed and did not parallel brain vascularity. She used biological assays (rat blood pressure-pressor response for NE and the rat uterus contractions for epinephrine) to measure tissue levels of "sympathin" (NE plus epinephrine). Also, the concentration of NE in the hypothalamus of cats was found to be unaffected by cervical sympathectomy. This suggested that the role of NE in the mammalian brain was more than as a neurotransmitter functioning at vascular smooth muscle nerve junctions.

Dopamine, on the other hand, was originally thought to be present in mammalian brain solely as a precursor of NE (Carlsson, 1966). However, it was found that DA was present in the mammalian whole brain in concentrations higher than NE (Montagu, 1957; Weil-Malherbe and Bone, 1957). Also, its distribution was found to differ markedly from that of NE (Cooper et al., 1974). These findings suggested that DA had functions other than being a precursor of NE.

Curtis and Crawford (1969) reviewed the findings that CA's applied iontophoretically to CNS neurons cause either facilitation or depression of neuronal firing. Bloom et al. (1965) found that DA and NE applied iontophoretically to neurons of the cat caudate nucleus predominantly caused depression of spontaneous unit discharge, although some were facilitated. Herz and Zieglgänsberger (1966) showed that iontophoretic DA was able to inhibit not only spontaneous activity of neurons in the rabbit caudate nucleus but also amino acid-induced discharges as well as discharges evoked by electrical stimulation of the thalamus.

Further suggestion of a neurotransmitter role of CA's in the CNS was the finding that CA's are continuously released from living brain tissue and that this release is increased by various stimuli (Glowinski, 1970). The release of DA from cat caudate nucleus was found to be increased by electrical stimulation of the centromedial nucleus of the thalamus (McLennan, 1964), by electrical stimulation of the substantia nigra (Portig and Vogt, 1969; von Voigtlander and Moore, 1971), and by application of K^+ or amphetamine (Besson et al., 1971).

Also, drugs that affect CA's in the CNS have been observed to modify brain function and behavior, again suggesting a neurotransmitter role of

CA's in the CNS. For example, reserpine has been found to cause sedation and tranquilization in human subjects, presumably by depleting the CA stores in the CNS (Goodman and Gilman, 1970). Likewise, Rech et al. (1966) observed that multiple intraperitoneal (i.p.) injections of α-methyl-para-tyrosine (αMPT), an inhibitor of CA synthesis, impaired avoidance response, rotarod performance, and spontaneous locomotor activity in rats at doses that did not produce obvious toxic effects. They found that the behavioral depression seen in rats correlated with the concentration of αMPT in the brain as well as with the depletion of NE and DA.

Adrenergic stimulation, direct and indirect, elicited by amphetamine, and to a lesser extent by ephedrine, has been found to cause tremor, restlessness, increased motor activity, agitation, and sleeplessness (Goodman and Gilman, 1970). Further, the antidepressant action of imipramine has been associated with its ability to block the uptake of NE at the synaptic membrane (Schildkraut, 1965; Schildkraut et al., 1971). Since reuptake of NE is the primary mechanism of deactivation of NE at the receptor (Iversen, 1971), blockade of reuptake would increase the concentration of NE at the synapse. Schildkraut (1965, 1974) suggested that affective disorders, such as manic-depression and depression, per se, are the result of a malfunction of the noradrenergic system in the brain. He suggested that mania is associated with an excess of NE, while depression is associated with a deficiency of that catecholamine. Imipramine is thought to ameliorate the symptoms of depression by increasing the concentration of NE at the synapse via a blockade of reuptake at the nerve terminal (Schildkraut et al., 1971).

Anatomical pathways of NE- and DA-containing neurons in mammalian CNS have been elucidated by Dahlström and Fuxe (1964) and Ungerstedt (1971),

using the fluorescent histochemical method of Carlsson, Falck, and Hillarp (1962). For example, a nigro-striatal dopaminergic pathway was found to originate in the substantia nigra and extend to the caudate nucleus (Andén et al., 1966). In addition, Fuxe (1965) found that the dopaminergic fibers originating in the substantia nigra also terminated in the putamen, the nucleus accumbens, and the tuberculum olfactorium.

Connor (1972) found that DA applied iontophoretically to neurons of the cat caudate nucleus caused depression of spontaneous neuronal firing. An identical response was elicited by electrical stimulation of the substantia nigra. α-methyldopamine (a pharmacological antagonist of DA), when applied continuously to a neuron, was found to block the effect of both iontophoretic DA and nigral stimulation on that neuron. This suggests that DA acts as a depressant neurotransmitter in the caudate nucleus and that it is released from the terminals of neurons originating in the substantia nigra.

In contrast, York (1970) found that iontophoretically applied DA caused facilitation of neuronal firing in the cat putamen. Electrical stimulation of the substantia nigra elicited a similar response. Therefore, an excitatory dopaminergic pathway from the substantia nigra to the putamen can be suggested.

Ungerstedt et al. (1969) found that the unilateral microinjection of DA into the caudate nucleus of awake freely moving rats caused asymmetric postural responses and turning to the contralateral side of application. Likewise, substances having direct or indirect dopaminergic activity, especially apomorphine, produced the same effect. Moreover, Ernst and Smelik (1966) found that implanted crystalline dopa (the immediate precursor of

DA) or apomorphine in various regions of rat striatum produced a compulsive gnawing behavior. The above findings indicate that striatal DA may be involved in motor function. Hornykiewicz (1966, 1972) stated that there is a large body of neuropharmacological evidence showing that the dopaminergic nigrostriatal system is directly involved in extrapyramidal control of motor function. He also stated that stereotyped behavior in rats, such as compulsive gnawing, is associated with the levels of DA in the striatum.

Isotopic and nonisotopic methods have been used to determine CA turnover (Costa and Neff, 1970). Brodie et al. (1966) used a nonisotopic method that utilized the rate of depletion of NE and DA after blockade of the rate-limiting step in CA biosynthesis via administration of αMPT. He determined the rate of depletion of CA's by measuring the levels of NE and DA at 0, 2, 4, 6, and 8 hours after administration of αMPT. Andén et al. (1969b) used the amount of NE and DA remaining 4 and 6 hours after administration of αMPT as an index of NE and DA turnover.

Costa and Neff (1970) found that the rate constant (fractional turnover rate) of NE efflux after blockade of NE synthesis with αMPT (nonisotopic method) was identical to the rate constant of the decline of NE-^{3}H after administration of tracer doses (isotopic method). They concluded that αMPT does not release NE, does not change the compartmentation of NE, and does not interfere with the enzymes that catabolize NE in vivo. The rate of depletion of NE and DA after synthesis blockade with αMPT was, therefore, concluded to be a valid indication of the turnover of NE and DA.

Moore and Dominic (1971) reviewed the effects of various stimuli on NE and DA turnover in mammalian brain. The literature revealed that stressful environmental stimuli, such as cold, exercise, immobilization, crowding,

and conditioned avoidance training cause an increase in NE turnover but had no effect on DA turnover. In addition, many drugs have been shown to alter the turnover of NE and DA. The turnover of DA is slowed by barbiturates and γ-hydroxybutyrate, while haloperidol and chlorpromazine (CPZ) increase NE and DA turnover (Andén et al., 1969b).

Andén et al. (1969b) suggested that turnover of CA's is correlated with the impulse activity of the catecholaminergic neurons. They found that electrical stimulation of the medulla oblongata causes an increase in the turnover of NE in the spinal cord. Also, transection of the spinal cord causes a decrease in the turnover of NE caudal to the section. Further, other findings indicate that the turnover of brain CA's is correlated with the impulse activity of the catecholaminergic neurons in the CNS as well. Cooper et al. (1974) stated that electrical stimulation of the locus coeruleus causes an increase in the turnover of NE as well as an increase in the accumulation of 3-methoxy-4-hydroxyphenyleneglycol-sulfate (MHPG-sulfate), the major metabolite of NE in the mammalian brain. Likewise, they stated that an increase in impulse flow in the nigro-neostriatal or mesolimbic DA systems leads to an increase in both DA turnover and the accumulation of homovanillic acid (HVA), the major metabolite of DA, in the striatum and the tuberculum olfactorium, respectively. (Cell bodies of the mesolimbic DA system are located just dorsal to the interpeduncular nucleus in the ventral tegmental area, and these cells innervate the nucleus accumbens and the tuberculum olfactorium. The function of the mesolimbic system is not known (Cooper et al., 1974).)

Parathion (O,O-diethyl-O-p-nitrophenyl phosphorothionate), a sulfur containing organophosphate, was first synthesized in 1944 by Gerhard

Schrader of I. G. Farbenindustrie in Germany. Many other organophosphates were synthesized during World War II by German and English researchers for use as nerve gases or as insecticides (O'Brien, 1967). Although "safer" insecticides possessing less mammalian toxicity, such as malathion, diazinon, and Sumithion, have since been developed, parathion continues to receive extensive use as an insecticide (Hybertson, 1971). In fact, parathion (parathion-ethyl, E605, Thiophos®, Alkron®, etc.) has become important throughout the world as a broad-spectrum insecticide (Lorenz and Sasse, 1968).

According to O'Brien (1967), acute toxicity to organophosphates in mammals is believed to be due to its anti-cholinesterase (anti-ChE) property. Phosphorothionates (such as parathion) are weak inhibitors of ChE (Aldridge and Barnes, 1952). But phosphorothionates are converted (activated) in the liver to potent ChE inhibitors; specifically, parathion is oxidized to paraoxon by microsomal enzymes in rat liver (see Nakatsugawa and Dahm (1967) for the chemical reaction). It is this metabolite of parathion that is a potent irreversible inhibitor of ChE (Mounter, 1963).

Paraoxon inhibits acetylcholinesterase (AChE), presumably by binding to the serine hydroxyl group of the enzyme. A serine hydroxyl group is essential for the normal function of the active site of AChE (Corbett, 1974). Holmstedt et al. (1967) found that inhibition of AChE activity in rat brain causes a marked increase in the endogenous concentration of ACh in the brain. It was, therefore, proposed that failure of AChE to hydrolyze ACh at the synapse causes an accumulation of ACh at the cholinergic "receptor" and, therefore, an enhancement of cholinergic stimulation (Fest and Schmidt, 1973).

Acetylcholine (ACh) is generally accepted as the neurotransmitter of the motor neuron axon collateral that synapses on the Renshaw cell in the spinal cord (see Cooper et al. (1974) for the review). The putative neurotransmitter role of ACh in mammalian brain has been extensively reviewed by Votava (1967), Pepeu (1972), and Baldessarini and Karobath (1973). ACh was first detected in mammalian brain by Chang and Gaddum (1933), who used a biological assay (eserinized frog rectus abdominis muscle) to determine the presence of ACh in rabbit brain. Dale (1934) suggested that ACh may act as a neurotransmitter in the peripheral nervous system. He ascribed the classical "muscarinic" and "nicotinic" actions to ACh. In brief, low doses of injected ACh elicit a response similar to that of muscarine, and higher doses of ACh elicit a response similar to that of nicotine. He also suggested the possible role of ACh as a neurotransmitter in the CNS. Feldberg and Vogt (1948) found that the CNS ACh-synthesis activity varied depending on the brain regions. For example, the caudate nucleus, the thalamus, and the lateral geniculate nucleus of the dog brain were found to possess the highest potential activity for ACh synthesis.

Ample evidence has accumulated to suggest that ACh is a neurotransmitter in mammalian brain. ACh and choline acetylase, the enzyme involved in the last step of ACh synthesis, were found to be localized in vesicles within synaptosomes from rat brain homogenate (De Robertis et al., 1962, 1963). They also found AChE present in the synaptosomes, but most of the activity was associated with the membranes of the nerve endings.

Phillis and Chong (1965) found that ACh was continuously released from the cerebral and cerebellar cortices. They observed that the amount of ACh release was increased by direct electrical stimulation of the cortex and by

stimulation of a variety of peripheral afferent pathways (limb, facial, and auditory stimulation). Also, electrical stimulation (30, 60, and 100 Hz) of the mesencephalic reticular formation enhanced the liberation of ACh from cat parietal cortex (Szerb, 1967). Celesia and Jasper (1966) found that ACh released from the somatosensory cortex of the cat was decreased during sleep and during barbiturate anesthesia but increased during electrical stimulation of the mesencephalic reticular system and during pentylenetetrazol-induced seizures. Also, Mitchell (1963) observed that chloralose blocked the release of ACh from the cortical surface and also blocked thalamic after-discharge following sensory stimulation. He further observed that the rates of ACh release from the cortex of the sheep, cat, and rabbit were roughly proportional to the spontaneous electrical activity of the brain. Based on Mitchell's and their own observations, Celesia and Jasper (1966) suggested that ACh may play a role in cortical activation of the type produced by the nonspecific (ascending reticular) activation system, a system distinct from the one involved in specific thalamocortical projection pathways.

Likewise, McLennan (1964) found that ACh was continuously released from the head of the caudate nucleus in the cat and that this release was increased by electrical stimulation of the ventral anterior thalamic nucleus. Further, McLennan and York (1966) found that microiontophoretic application of ACh to neurons in the caudate nucleus of the cat caused excitation in some neurons and inhibition in others. The two types of neurons were found to be localized in different regions of the caudate nucleus. With each type of neuron, a similar response was noted during electrical stimulation of the ventral anterior thalamic nucleus. Both

responses, either that elicited by ACh application or that elicited by electrical stimulation of the thalamic nucleus, were blocked by atropine (McLennan and York, 1966). McLennan (1970) suggested a cholinergic pathway that originates in the anterior thalamus and innervates (either in an excitatory or inhibitory manner) neurons in the caudate nucleus.

Votava (1967) and Curtis and Crawford (1969) have reviewed the direct effect of microiontophoretically applied cholinomimetics on various regions of the mammalian CNS. Cholinoceptive neurons in the cortex were found to be primarily "muscarinic," while a "nicotinic" response was elicited from the Renshaw cell. Cholinoceptive neurons in the thalamus responded in both a "nicotinic" and "muscarinic" manner. Bradley and Wolstencroft (1965) found that the receptors of the cholinoceptive neurons in the brain stem were of both an excitatory and inhibitory nature. The inhibitory receptors appeared to be exclusively "muscarinic," whereas the excitatory receptors had both "muscarinic" and "nicotinic" properties.

Monnier and Romanowski (1962) found that injection of ACh, physostigmine (a reversible ChE inhibitor), or pilocarpine (a cholinomimetic) into the carotid artery of the rabbit caused an EEG arousal syndrome characterized by desynchronized electrical activity in the neocortex with synchronization in the hippocampus, caudate nucleus, thalamus, and midbrain reticular system. They also observed a decrease in the recruiting potentials evoked by electrical stimulation of the medial thalamus. This suggests that cholinoceptive neurons may be involved in multiple functions of the CNS.

Holmstedt and Lundgren (1966) found that administration of oxotremorine and arecoline (both cholinomimetics) in rats caused a rise in the ACh con-

centration as well as tremor. The tremor appeared to be dependent upon the concentration of ACh in the brain. Also, they found the tremor activity to be muscarinic in nature, since administration of atropine (a muscarinic blocker) was found to abort the tremor. This suggests the involvement of CNS cholinergic (muscarinic) neurons in motor function.

One of the first indications of an interaction between the catecholaminergic and cholinergic system in mammalian brain was the observation that anticholinergics (antimuscarinics), such as atropine and scopolamine, could ameliorate some of the symptoms of Parkinson's disease (paralysis agitans), a disease of the extrapyramidal motor system associated with a malfunction of dopaminergic neurons in the CNS (Hornykiewicz, 1966, 1972).

Ehringer and Hornykiewicz (1960) found the levels of DA in the caudate nucleus, putamen, and globus pallidus to be much lower in parkinsonian patients than in nonparkinsonian humans. The concentration of HVA in the caudate nucleus, putamen, substantia nigra, and globus pallidus (Bernheimer and Hornykiewicz, 1964, 1965) and in the cerebrospinal fluid (Johansson and Roos, 1967) were also found to be reduced in parkinsonian patients. In addition, Bernheimer and Hornykiewicz (1964, 1965) found a reduced turnover of DA in the brain of parkinsonian patients, and they suggested that this was probably the consequence of an impairment of DA formation.

Pathological lesions in the substantia nigra and neostriatum have been found to be the most typical morphological abnormalities associated with parkinsonism (Selby, 1968; Turner, 1968; Calne and Sandler, 1970). Experimentally-induced lesions of the substantia nigra diminish DA distally to the lesions and produced tremor and akinesia (typical symptoms of parkinsonism) (Sourkes and Poirier, 1966).

Some phenothiazines, such as chlorpromazine (CPZ), and butyrophenones, such as haloperidol, have produced extrapyramidal syndromes in man with many of the symptoms of Parkinson's disease (Delay and Deniker, 1968). York (1972) suggested that the mechanism of drug-induced parkinsonism involved the blockade of DA receptors in the brain. Both CPZ and haloperidol have been shown to block DA receptors in experimental animals (van Rossum, 1966; Yeh et al., 1969).

Anticholinergic drugs (primarily the belladonna alkaloids) have been the "mainstay" of drug therapy of parkinsonism for over 100 years (Selby, 1968). A crude preparation of belladonna alkaloids, containing hyoscine (scopolamine), was observed to "promptly mitigate" the severity of tremor and tended to relax muscular rigidity associated with Parkinson's disease (Yahr and Duvoisin, 1968). Also, anticholinergics have been found to be of value in the treatment of drug-induced extrapyramidal disorders (Delay and Deniker, 1968).

Shute and Lewis (1966) stated that the neostriatum is a structure in which there is a functional overlap of the cholinergic and catecholaminergic systems and that these systems may exert a mutual antagonism. McGeer et al. (1961) and Barbeau (1962) suggested that normal extrapyramidal motor function depends upon a sensitive balance between inhibitory dopaminergic neurons and excitatory cholinergic neurons in the striatum. If the dopaminergic system in the brain is deficient, as in parkinsonian, the cholinergic system will dominate.

Coyle and Snyder (1969) found that a variety of antiparkinsonian drugs (benztropine, trihexyphenidyl, diphenhydramine, orphenadrine, phenindamine, and diethazine) are potent noncompetitive inhibitors of DA uptake into syn-

aptosomes isolated from rat corpus striatum. This suggests an alternative mechanism of action for drugs previously thought to act by muscarinic antagonism. However, cholinomimetics, such as physostigmine and ACh, have been shown to exacerbate parkinsonian symptoms (Barbeau, 1974), again adding support to the proposed involvement of cholinergic neurons in extrapyramidal motor function.

Other findings indicate an interaction of cholinomimetic drugs with catecholaminergic neurons in the CNS. Van Meter and Karczmar (1971) found that the physostigmine-induced attenuation of the thalamocortical recruitment response in rabbit brain was dependent on the CA levels. They found that depletion of CA's in rabbit brain by pretreatment with reserpine or αMPT prevented the physostigmine-induced response.

Burn and Rand (1965) as well as Ferry (1966) reviewed the interaction of cholinergic and adrenergic nervous systems (primarily in peripheral nerves). They proposed a mechanism for the involvement of ACh in the release of NE from the sympathetic postganglionic nerve terminals (the cholinergic link hypothesis). These authors suggested that both ACh and NE are present in the same neuron and that a nerve impulse reaching the axon terminal initiates the release of ACh. After its release, ACh binds to "nicotinic" receptors at the presynaptic membrane causing an influx of calcium ions, which in turn triggers the release of NE. However, no unequivocal evidence has been obtained for the simultaneous presence of NE- and ACh-containing vesicles in one and the same nerve terminals (Kosterlitz and Lees, 1972).

For a more extensive review of the interactions of catecholaminergic and cholinergic neurons in the mammalian brain, see Izquierdo (1972).

METHODS AND MATERIALS

Part A. Comparison of Two Experimental Designs for the Determination of NE and DA Turnover

In the first part of this study, two experimental designs (Design 1 and Design 2) were used to determine turnover of NE and DA in selected regions of rat brain.

Design 1

Male hooded rats[1] (300-500 g) were used as the experimental animal. The rats were given feed and water ad lib. and were subjected to a lighting schedule of 6 A.M.-6 P.M. of light and 6 P.M.-6 A.M. of darkness. Four rats were randomly assigned to each of 5 time groups (0, 2, 4, 6, and 8 hour groups). Rats in the 2, 4, 6, and 8 hour groups were given α-methyl-para-tyrosine[2] (200 mg/kg, i.p.) in a volume of 2 ml/kg. Rats in the 4, 6, and 8 hour groups were administered a second dose of αMPT (100 mg/kg, i.p.) in a volume of 2 ml/kg 2.5 hours after the first dose. αMPT was made up as a solution in double-distilled water (d.d. H_2O). Control rats (rats in the 0 hour group) were given saline (2 ml/kg, i.p.). All rats were administered atropine methylnitrate (0.3 mg/kg, i.p.) 20 minutes prior to the administration of αMPT or saline. Atropine methylnitrate (AMN) was made up as a solution in saline and was given in a volume of 1 ml/kg to antagonize the peripheral parasympathetic nervous system dominance in the αMPT-treated rats.

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[1] Long-Evans descent.

[2] α-methyl-para-tyrosine(methyl ester)-HCL from Regis Chemical Co.

All injections (in this part and in subsequent parts of the study) were made within a 2-hour interval between 9 A.M. and 11 A.M. The variations of NE and DA levels that follow a circadian rhythm in rat brain are minimal throughout this time period (Scheving et al., 1968).

Control rats were sacrificed within one hour after the injection of saline. αMPT-treated rats were sacrificed at 2, 4, 6, and 8 hours after administration of the first dose of αMPT. All sacrifices were made by decapitation followed by (a) removal of the brain, (b) dissection of the brain into the selected regions (cerebral cortex, brain stem, hypothalamus, and corpus striatum), and (c) placement of the brain parts into liquid nitrogen for storage. The above procedure (from the decapitation to step (c)) was completed in less than 5 minutes for each rat. Brain regions were pooled from all 4 rats in a given time group. The NE and DA levels of the pooled samples were determined fluorometrically (see Appendix B).

A least squares fitting of linear regression of log[CA] versus time was made for NE and DA in each brain region. Statistically significant differences between rate constants were determined from the slopes of the regression lines. For a more detailed description of Design 1, see Appendix C.

Design 2

The total experiment using Design 2 was composed of 3 or 4 individual experiments carried out on separate days. In each individual experiment, 2 to 4 male hooded rats (200-450 g) were used for a control group and a similar number of rats for an αMPT-treated group. The rats were given feed, water, and a lighting schedule the same as in Design 1.

Rats in the treated group were administered αMPT (200 mg/kg, i.p.) in a volume of 2 ml/kg and were sacrificed 4 hours later. Control rats were given saline (2 ml/kg, i.p.) and sacrificed within 1 hour. The brains were removed immediately after sacrifice, dissected, and analyzed for NE and DA as described in Design 1. The turnover of NE and DA in each brain region was determined from the slope of the line connecting the mean log[CA] at 0 hour (the control) and 4 hours (the αMPT treatment) after administration of αMPT. For a more detailed description of Design 2, see Appendix C.

Part B. Effects of Parathion on Steady-State Levels and Turnover of NE and DA in Selected Regions of Rat Brain

Steady-state levels

Male hooded rats (200-500 g) were randomly assigned to one of two treatment groups (control and parathion treatment). The rats were given feed, water, and a lighting schedule the same as in Part A (see above). Only male rats were used in this study, since the susceptibility of rats to parathion treatment had been shown to be dependent upon sex, i.e. female rats are approximately 2 times more susceptible to parathion toxicity than male rats (DuBois et al., 1949).

Parathion-treated rats received parathion[1] (1.25-2.50 mg/kg, i.p.) in a volume of 2 ml/kg. Triethylene glycol was used as the vehicle. The dose of parathion was varied from 1.25 to 2.50 mg/kg in order to find a dose that would be sublethal, yet give observable toxic signs. The LD50 of parathion given i.p. to adult male Holtzman rats has been determine to be

[1] Parathion (98% pure) was obtained from Chem Service, Inc.

3.6 mg/kg (Brodeur and DuBois, 1963). Control rats were given triethylene glycol (2 ml/kg, i.p.). All rats were given AMN (0.3 mg/kg, i.p.) 20 minutes prior to the administration of parathion or triethylene glycol. All rats were sacrificed 4 hours after the administration of parathion or triethylene glycol. The brain was removed, dissected, and analyzed for NE and DA as previously described. In addition, the hippocampus was dissected out and stored in liquid nitrogen for later use in the determination of the percent inhibition of brain ChE. Brain ChE activity was determined by a pH-stat method (see Appendix D).

Three replications of the experiment were carried out on separate days. The data were analyzed for significant differences between the levels of NE (or DA) in the control and parathion-treated rats for each brain region. The data were blocked according to replicates (days), for the statistical analysis, since the steady-state levels of NE and DA were observed to vary considerably from day to day. The mathematical model for the statistical analysis of the data was as follows:

$$Y_{ijk} = \mu + \pi_i + \beta_j + \gamma(X_{ijk} - \bar{X}_{...}) + \pi\beta_{ij} + e_{ijk}$$ [1]

where Y_{ijk} is the concentration of CA,

μ is the population mean of CA concentrations,

π_i is the treatment effect,

β_j is the block (day) effect,

γ is the regression coefficient of Y on X,

X_{ijk} is the covariate (body weight of the rat),

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[1] The mathematical model was adapted from Cochran and Cox (1957) and Snedecor and Cochran (1967).

$\bar{X}_{...}$ is the overall mean of rat body weight,

$\pi\beta_{ij}$ is the interaction of the π and β effects,

and e_{ijk} is the error term.

Since γ and $\pi\beta_{ij}$ were not found to be significant, the model was reduced to:

$$Y_{ijk} = \mu + \pi_i + \beta_j + e_{ijk}$$

Turnover

The experimental design for the determination of the effects of parathion on NE and DA turnover is based on Design 2 (see above). Male hooded rats (200-450 g) were randomly assigned to one of three treatment groups: control, αMPT treatment, and αMPT plus parathion treatment. Rats in the αMPT plus parathion treatment group were administered αMPT (200 mg/kg, i.p.) at the same time as parathion (1.25-2.50 mg/kg, i.p.). The volumes of administered αMPT and parathion were each 2 ml/kg, and the solutions were prepared as previously described. Rats in the αMPT treatment group were administered αMPT (200 mg/kg, i.p.) at the same time as triethylene glycol. Both were given in volumes of 2 ml/kg. Rats in the control group were given saline and triethylene glycol in volumes of 2 ml/kg. Atropine methylnitrate (0.3 mg/kg, i.p.) was given to all rats 20 minutes prior to the administration of the treatment drugs or their vehicles.

Control rats were sacrificed within 1 hour after injection of saline. αMPT- and αMPT plus parathion-treated rats were sacrificed 4 hours after administration of the treatment drugs. The brains were removed after decapitation, dissected, and analyzed for NE and DA as previously described. The hippocampus was analyzed for ChE activity using a

pH-stat method (see Appendix D). Percent inhibition of brain ChE was determined from the ChE activity in the hippocampus of parathion-treated rats as compared with control rats. Three or four replications of the experiment were carried out on separate days. The data were blocked according to replicates (days) for the statistical analysis. Significant differences between the effects of αMPT and αMPT plus parathion treatments on the depletion of NE and DA were determined using a statistical analysis based on the mathematical model described for the steady-state levels study (see above).

RESULTS

Figure 1 shows the graph of log[NE] versus time for the hypothalamus, brain stem, and cortex of the adult male hooded rat after administration of αMPT (200 mg/kg, i.p.). The linear regression of log[NE] versus time was found to be significant for all brain regions: hypothalamus ($P<0.005$), brain stem ($P<0.001$), and cortex ($P<0.005$). In addition, the slopes of the regression lines were found to be significantly different from each other. This was used to determine the significant differences between rate constants of NE in the various brain regions (rate constant = $-b/0.4343$, where b is the slope of the regression line).

Table 1 shows the steady-state levels, rate constants, turnover times, and turnover rates of NE in selected regions of rat brain using Design 1. Note that significant differences were found between the rate constants of NE in: cortex vs. brain stem ($P<0.025$), cortex vs. hypothalamus ($P<0.005$), and hypothalamus vs. brain stem ($P<0.050$).

Similarly, Table 2 shows the steady-state levels, rate constants, turnover times, and turnover rates of DA in the cortex and corpus striatum of rat brain using Design 1. However, no significant differences were found between the rate constants.

Tables 3 and 4 show the steady-state levels, rate constants, turnover times, and turnover rates of NE and DA in selected regions of rat brain obtained using Design 1 compared with Design 2. The rate constants obtained for the two experimental designs are essentially in agreement. However, a larger discrepancy between Design 1 and Design 2 is noted for the steady-state levels and turnover rates of NE and DA in the various

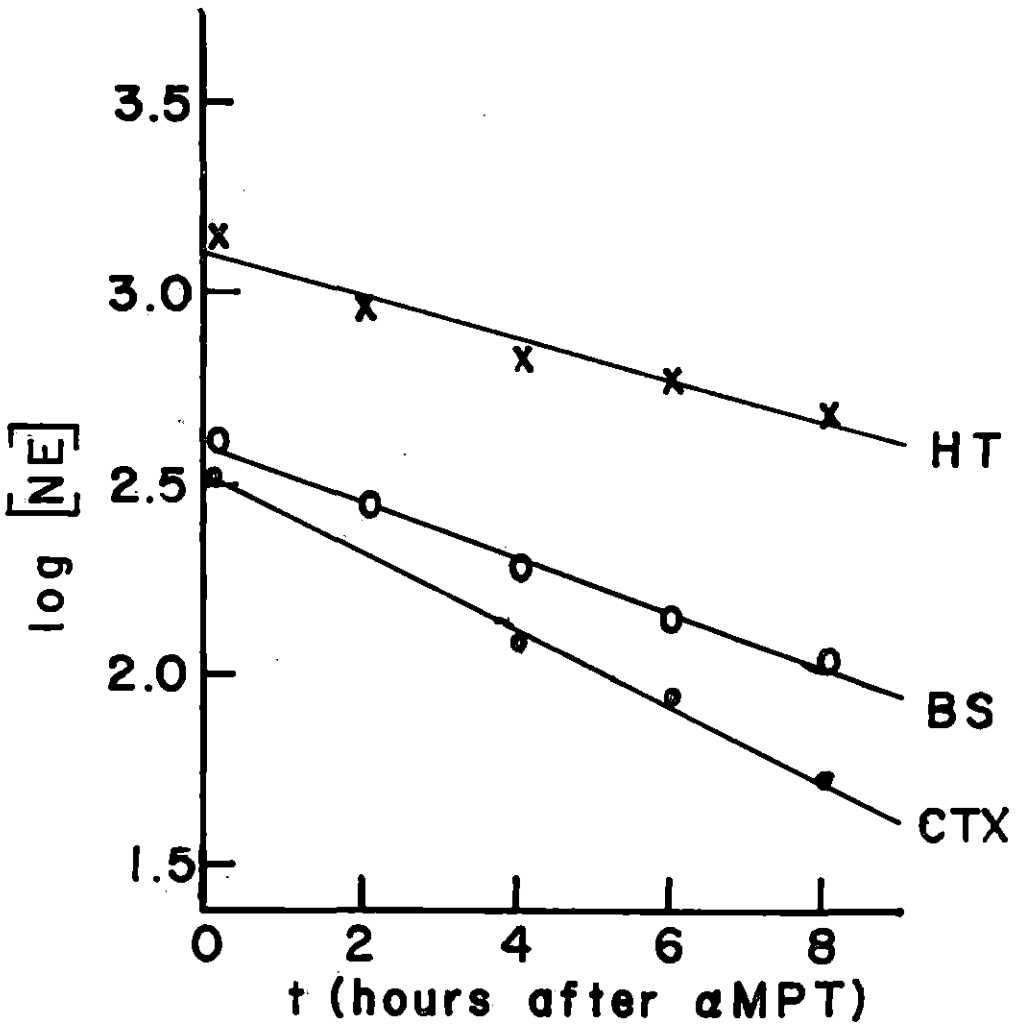

Figure 1. Graph of log[NE] (NE in ng/g) versus t (hours after administration of αMPT) for three rat brain regions: hypothalamus (HT), brain stem (BS), and cortex (CTX)

The linear regression of log[NE] versus time was found to be significant for all brain parts: HT ($P<0.005$), BS ($P<0.001$), and CTX ($P<0.005$). The slopes of the regression lines for the three brain regions were found to be significantly different from each other.

Table 1. Steady-state levels, rate constants, turnover times, and turnover rates of NE[a] in selected regions of rat brain determined by Design 1

Brain regions	SSL[b] (ng/g)	k[c] (hr^{-1})	tt (hr)	K (ng/g/hr)
CTX	314	0.218 ± 0.013 (4)	4.59	68.5
BS	391	0.166 ± 0.0085[d] (5)	6.03	64.8
HT	1230	0.123 ± 0.012[e] (5)	8.13	151

[a]Abbreviations: SSL = steady-state level, k = rate constant, tt = turnover time, K = turnover rate, CTX = cortex, BS = brain stem, HT = hypothalamus.

[b]The SSL was determined from the y-intercept of the graph of log[NE] versus time.

[c]The rate constant ± standard error was calculated from the slope of the regression of log[NE] versus time. The number in parentheses was the number of observations.

[d]Significantly different from k for the cortex ($P<0.025$) and for the hypothalamus ($P<0.050$).

[e]Significantly different from k for the cortex ($P<0.005$).

brain regions. The steady-state levels of NE and DA in rat brain were observed to vary noticeably from day to day and between groups of animals.

Table 5 shows the effects of parathion treatment on the steady-state levels of NE and DA in selected regions of rat brain (cortex, brain stem, and corpus striatum). All parathion treated rats were included in the treatment group. Inhibition of ChE in the brain (hippocampus) of the treated rats was observed to vary from 0% to 80%. The effects of parathion treatment on steady-state levels of NE and DA in the selected regions of rat brain were found to be not significant, except for NE in the cortex,

Table 2. Steady-state levels, rate constants, turnover times, and turnover rates of DA in selected regions of rat brain determined by Design 1[a]

Brain regions	SSL[b] (ng/g)	k_{-1}[c] (hr^{-1})	tt (hr)	K (ng/g/hr)
CTX	575	0.280 ± 0.034 (3)	3.57	161
CS	13,800	0.210 ± 0.014[d] (5)	4.75	2900

[a]Abbreviations: SSL = steady-state level, k = rate constant, tt = turnover time, K = turnover rate, CTX = cortex, CS = corpus striatum.

[b]The SSL was determined from the y-intercept of the graph of log[NE] versus time.

[c]The rate constant ± standard error was calculated from the slope of the regression of log[DA] versus time. The number in parentheses was the number of observations.

[d]No significant difference between the rate constants of DA in the cortex and corpus striatum.

which was significantly decreased ($P<0.025$) as measured 4 hours after administration of parathion.

Table 6 includes only parathion treated animals in which brain ChE was inhibited greater than 50%. The steady-state level of NE in the cortex was significantly decreased by parathion treatment ($P<0.03$). All other steady-state levels were not significantly affected by parathion treatment.

Table 7 shows the effects of parathion treatment on the depletion of NE in selected regions of the brain of the adult male hooded rat as measured 4 hours after administration of αMPT. The first grouping of parathion treatment (parathion, inh. = 0-80%) included all rats given parathion, in which 0% to 80% inhibition of brain ChE was observed. The second grouping

Table 3. Steady-state levels, rate constants, turnover times, and turnover rates of NE in selected regions of rat brain determined by Design 1 and Design 2[a]

Brain regions	SSL (ng/g) D1[b]	SSL (ng/g) D2[c]	k (hr^{-1}) D1	k (hr^{-1}) D2	tt (hr) D1	tt (hr) D2	K (ng/g/hr) D1	K (ng/g/hr) D2
CTX	314	279 ± 12 (10)	0.218 ± 0.013 (4)	0.224 ± 0.026 (20)	4.59	4.45	68.5	62.6 ± 7.7
BS	391	507 ± 15 (10)	0.166 ± 0.0085 (5)	0.195 ± 0.014 (20)	6.03	5.13	64.8	98.9 ± 7.7

[a]Abbreviations: SSL = steady-state level, k = rate constant, tt = turnover time, K = turnover rate, D1 = Design 1, D2 = Design 2, CTX = cortex, BS = brain stem.

[b]cf. Table 1.

[c]Values for Design 2 are reported as the mean ± standard error of the mean. The number in parentheses was the number of observations.

Table 4. Steady-state levels, rate constants, turnover times, and turnover rates of DA in selected regions of rat brain determined by Design 1 and Design 2[a]

Brain regions	SSL (ng/g)		K (hr^{-1})		tt (hr)		K (ng/g/hr)	
	D1[b]	D2[c]	D1	D2	D1	D2	D1	D2
CTX	575	461 ± 48 (10)	0.280 ± 0.035 (5)	0.299 ± 0.044 (20)	3.57	3.35	161	138 ± 25
CS	13,790	9360 ± 570 (12)	0.210 ± 0.013 (5)	0.211 ± 0.025 (24)	4.76	4.74	2900	1980 ± 280

[a]Abbreviations: SSL = steady-state level, k = rate constant, tt = turnover time, K = turnover rate, D1 = Design 1, D2 = Design 2, CTX = cortex, CS = corpus striatum.

[b]cf. Table 2.

[c]Values for Design 2 are reported as the mean ± standard error of the mean. The number in parentheses was the number of observations.

Table 5. Effects of parathion on steady-state levels of NE and DA in selected regions of rat brain

Treatment	Cortex		Brain stem		Corpus striatum	
	[NE] (ng/g)	[DA] (ng/g)	[NE] (ng/g)	[DA] (ng/g)	[NE] (ng/g)	[DA] (ng/g)
Control[a]	307±7.7 (14)[b]	527±27 (14)	535±8.5 (15)	198±16 (15)	151±10 (14)	8595±506 (14)
Parathion[c]	282±6.2 (22)[d]	442±22 (21)	526±7.4 (20)	217±13 (21)	148±8.7 (20)	9391±423 (20)

[a]Control rats were given an equivalent volume of triethyleneglycol, the vehicle of parathion.

[b]Mean CA concentration ± standard error of the mean. The number in parentheses was the number of observations.

[c]Parathion (1.25-2.50 mg/kg, i.p.) was given as a 0.125% solution in triethyleneglycol. Rats were sacrificed 4 hours after administration of parathion.

[d]Significantly different from control [NE] ($P<0.025$).

Table 6. Effects of parathion (with brain ChE inhibited greater than 50%) on steady-state levels of NE and DA in selected regions of rat brain

Treatment	Cortex		Brain stem		Corpus striatum	
	[NE] (ng/g)	[DA] (ng/g)	[NE] (ng/g)	[DA] (ng/g)	[NE] (ng/g)	[DA] (ng/g)
Control[a]	300±9.2 (10)[b]	489±32 (10)	516±9.9 (11)	192±18 (11)	159±12 (10)	9157±599 (10)
Parathion[c]	277±9.6 (9)[d]	467±33 (9)	506 ±11 (9)	195±20 (9)	132±11 (8)	11020±670 (8)

[a]Control rats were given an equivalent volume of triethyleneglycol, the vehicle of parathion.

[b]Mean CA concentration ± standard error of the mean. The number in parentheses was the number of observations.

[c]Parathion (1.25-2.50 mg/kg, i.p.) was given as a 0.125% solution in triethyleneglycol. Rats were sacrificed 4 hours after administration of parathion.

[d]Significantly different from control [NE] ($P<0.03$).

Table 7. Effects of parathion[a] on the depletion of NE in selected regions of rat brain after administration of αMPT[b]

Treatment	Cortex [NE] (ng/g)	Cortex % control [NE]	Brain stem [NE] (ng/g)	Brain stem % control [NE]
Control[c]	279 ± 12 (10)[d]	100.	507 ± 15 (10)	100.
αMPT	115 ± 7.4 (10)	41.1	233 ± 7.1 (10)	45.8
αMPT + parathion (inh. = 0-80%)[e]	117 ± 8.0 (12)	42.0	217 ± 15 (11)	42.9
αMPT + parathion (inh. > 50%)[f]	108 ± 13 (6)	38.6	198 ± 24 (6)	39.2
αMPT + parathion (inh. > 60%)[g]	91.7 ± 8.0 (4)	32.9	171 ± 24 (4)[h]	33.6

[a]Parathion (1.25-2.50 mg/kg, i.p.) was given as a 0.125% solution in triethyleneglycol.

[b]αMPT-treated rats were sacrificed 4 hours after administration of αMPT (200 mg/kg, i.p.).

[c]Control rats were given an equivalent volume of triethyleneglycol, the vehicle of parathion, and saline.

[d]Mean NE concentration ± standard error of the mean. The number in parentheses was the number of observations.

[e]Included all rats in which parathion caused 0-80% inhibition of ChE.

[f]Included only rats in which parathion caused > 50% inhibition of ChE.

[g]Included only rats in which parathion caused > 60% inhibition of ChE.

[h]Significantly different from [NE] in αMPT treatment ($P<0.005$).

of parathion treatment (parathion, inh. > 50%) included only rats in which brain ChE was inhibited greater than 50%. The third grouping of parathion treatment (parathion, inh. > 60%) included only rats in which brain ChE was inhibited greater than 60%.

No significant differences were found between [NE] after the αMPT + parathion (inh. = 0-80%) treatment and [NE] after the αMPT treatment for both cortex and brain stem. Likewise, no significant differences were found between [NE] after the αMPT + parathion (inh. > 50%) treatment and [NE] after the αMPT treatment for both cortex and brain stem. However, a significant difference was found between [NE] after the αMPT + parathion (inh. > 60%) treatment and [NE] after the αMPT treatment for the brain stem. The level of NE remaining 4 hours after administration of αMPT in the rat brain stem was significantly lowered by parathion, which had caused inhibition of brain ChE greater than 60%. No significant difference was found, however, between [NE] following the αMPT + parathion (inh. > 60%) treatment and [NE] after the αMPT treatment for the cortex. Part of the above data is graphically represented in Figure 2. Note that the above data suggest a dependence of the NE depletion induced by αMPT in the brain stem upon the percent inhibition of brain ChE.

Table 8 shows the effects of parathion treatment on the depletion of DA in the cortex and corpus striatum of the adult male hooded rat. Parathion (inh. = 0-80%) and (inh. > 50%) significantly altered the levels of DA remaining in the cortex and corpus striatum 4 hours after administration of αMPT. However, the parathion treatment had opposite effects on the depletion of DA in the two brain regions. The level of DA in the cortex 4 hours after administration of αMPT was significantly increased by para-

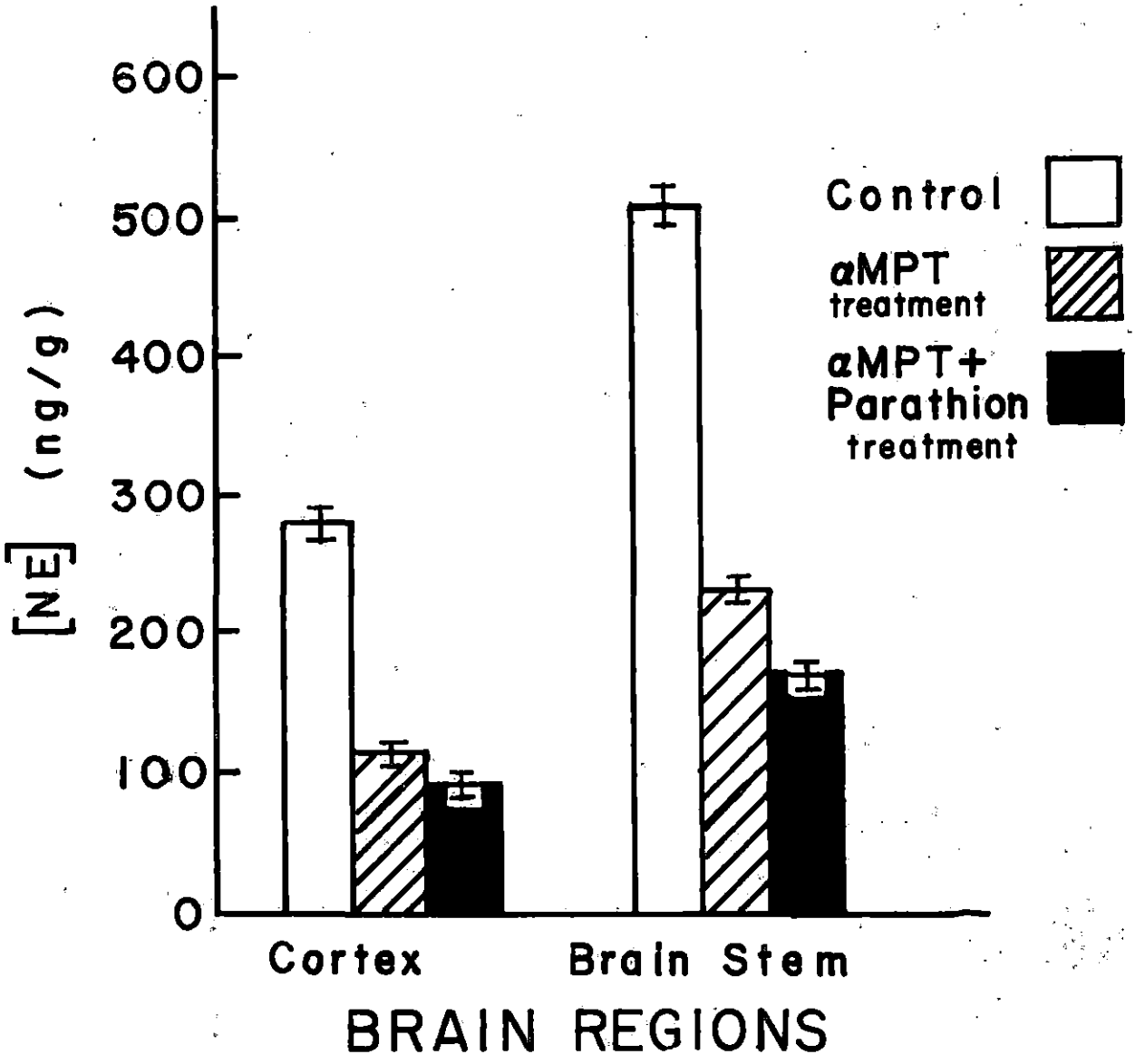

Figure 2. Mean concentration of NE in the cortex and brain stem of control rats, rats 4 hours after administration of αMPT, and rats 4 hours after administration of αMPT plus parathion

Only rats, in which brain ChE was inhibited greater than 60%, were included in the third treatment group. The standard errors of the means are indicated.

Table 8. Effects of parathion[a] on the depletion of DA in selected regions of rat brain after administration of αMPT[b]

Treatment[c]	Cortex [DA] (ng/g)	% control [DA]	Corpus striatum [DA] (ng/g)	% control [DA]
Control	461 ± 48 (10)[d]	100.	9360 ± 570 (12)	100.
αMPT	137 ± 11 (10)	29.8	3980 ± 170 (12)	42.5
αMPT + parathion (inh. = 0-80%)	179 ± 15 (11)[e]	38.8	3490 ± 130 (18)[f]	37.3
αMPT + parathion (inh. > 50%)	165 ± 22 (6)[g]	35.8	3360 ± 150 (12)[h]	35.9

[a]Parathion (1.25-2.50 mg/kg, i.p.) was given as a 0.125% solution in triethyleneglycol.

[b]αMPT-treated rats were sacrificed 4 hours after administration of αMPT (200 mg/kg, i.p.).

[c]Treatments were the same as described in Table 7.

[d]Mean DA concentration ± standard error of the mean. The number in parentheses was the number of observations.

[e]Significantly different from [DA] in αMPT treatment ($P<0.005$).

[f]Significantly different from [DA] in αMPT treatment ($P<0.02$).

[g]Significantly different from [DA] in αMPT treatment ($P<0.02$).

[h]Significantly different from [DA] in αMPT treatment ($P<0.03$).

thion treatment. In contrast, the level of DA in the corpus striatum after αMPT was significantly lower subsequent to parathion treatment. Part of the above data is graphically represented in Figure 3.

Figure 4 shows the graph of percent control [NE] in the brain stem versus percent inhibition of brain ChE. The depletion of NE in the brain stem after αMPT appears to be increased by higher values of percent

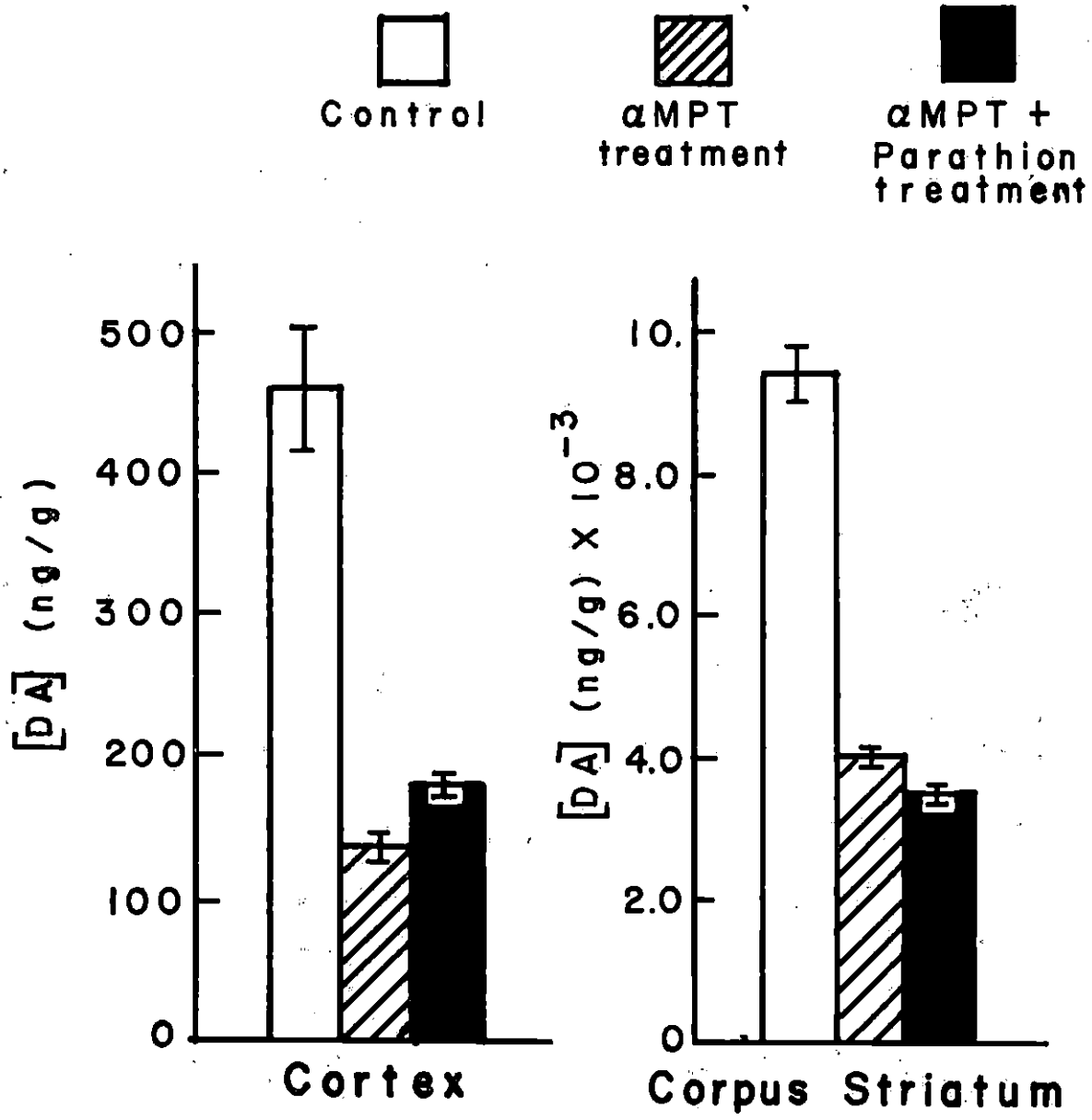

Figure 3. Mean concentration of DA in the cortex and corpus striatum of control rats, rats 4 hours after administration of αMPT, and rats 4 hours after administration of αMPT plus parathion

All rats given parathion, in which brain ChE was inhibited from 0 to 80%, were included in the third treatment group. The standard errors of the means are indicated.

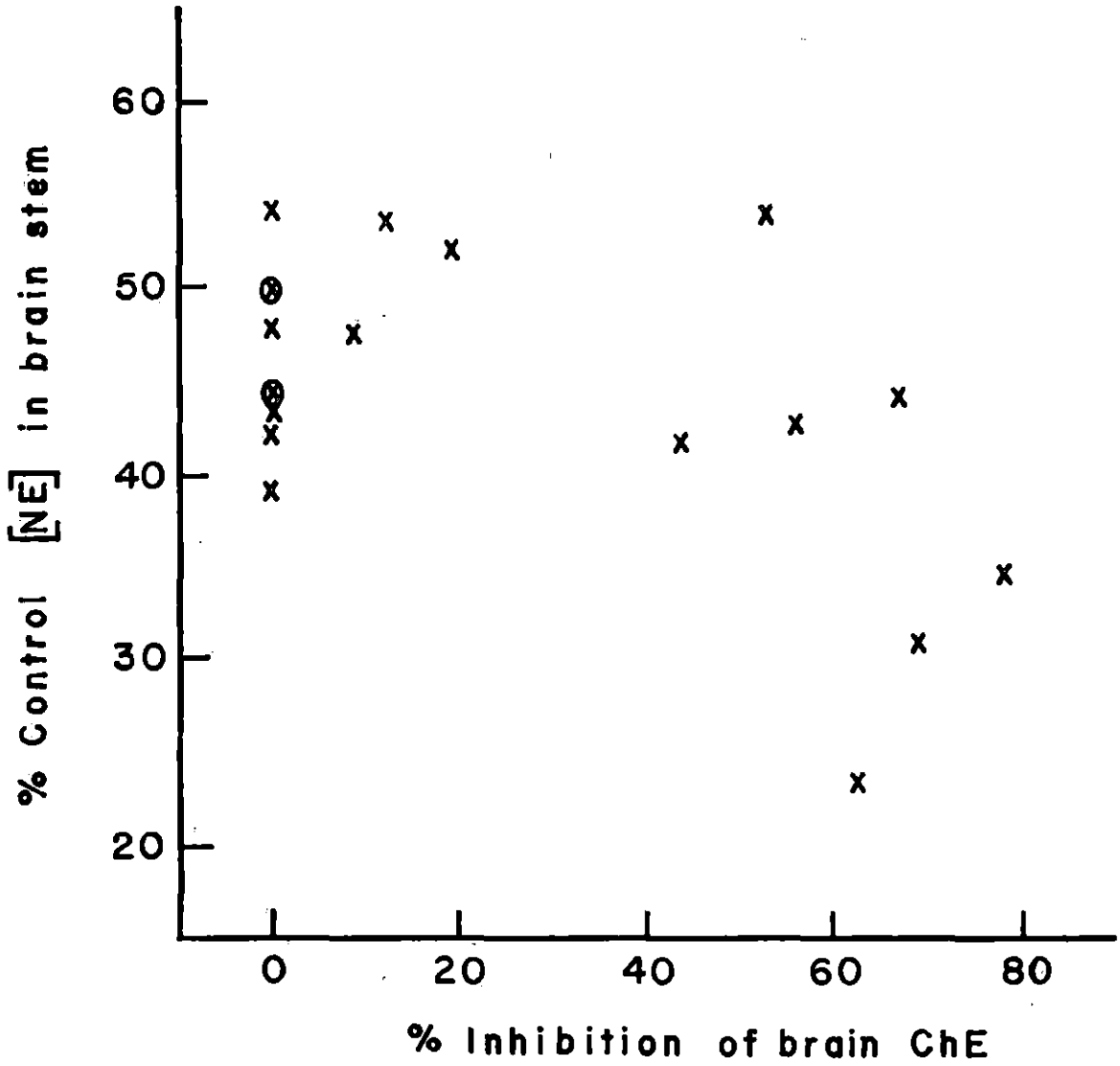

Figure 4. Graph of percent control [NE] in rat brain stem 4 hours after administration of αMPT versus percent inhibition of brain ChE caused by parathion treatment. Values at 0% inhibition of brain ChE were from rats given only αMPT

inhibition of brain ChE. This suggests a dependence of NE depletion in the brain stem upon the percent inhibition of brain ChE. Likewise, Figure 5 shows the graph of percent control [NE] in the cortex after αMPT versus percent inhibition of brain ChE. The depletion of NE in the cortex after αMPT appears to be increased by higher values of percent inhibition of brain ChE, again suggesting the dependence of NE depletion after αMPT upon the percent inhibition of brain ChE.

Figures 6 and 7 show the graphs of percent control [DA] in the cortex and corpus striatum versus percent inhibition of brain ChE. No definite pattern can be distinguished. The depletion of DA in the cortex and corpus striatum does not appear to be dependent upon the percent inhibition of brain ChE.

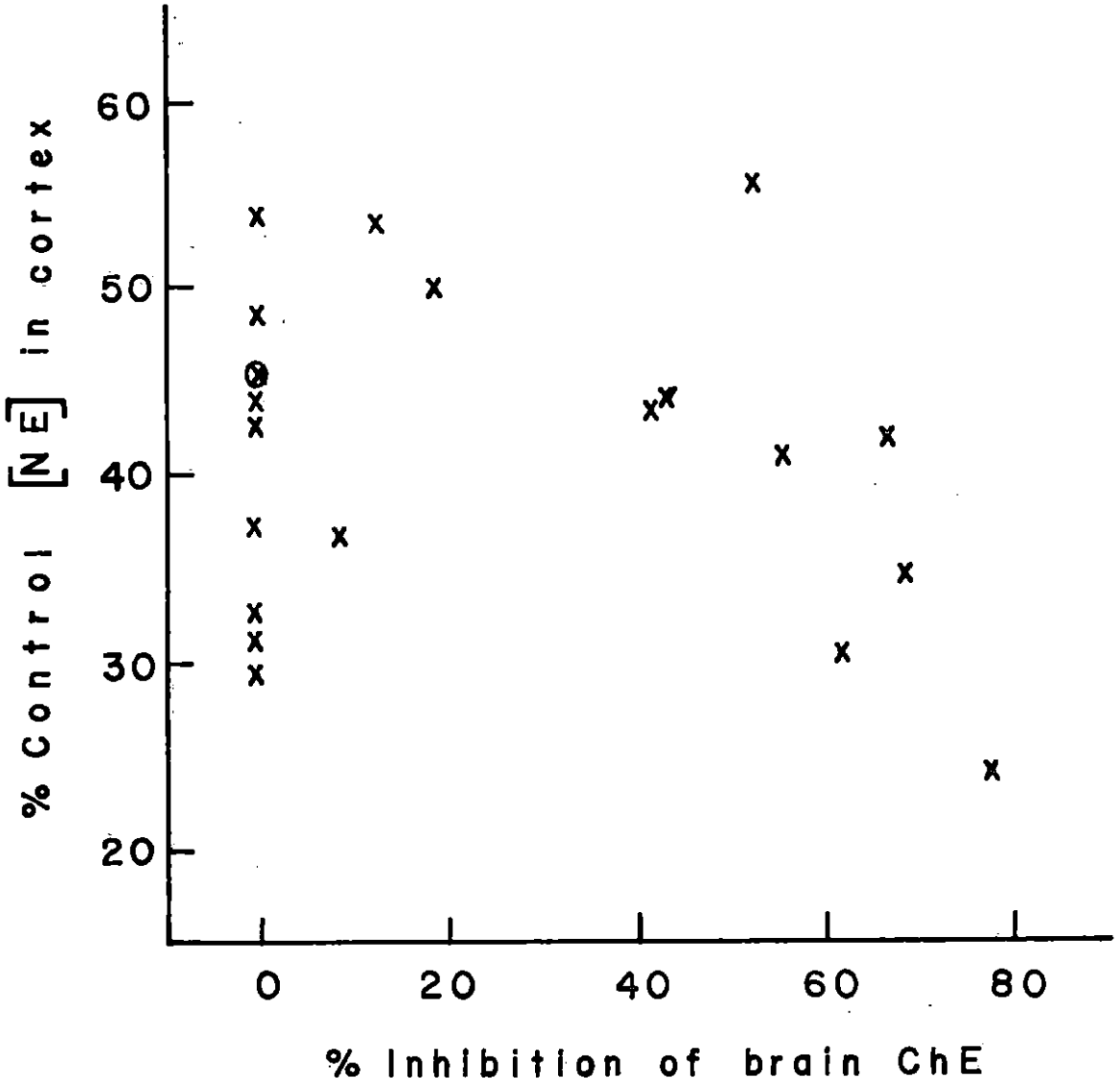

Figure 5. Graph of percent control [NE] in rat cortex 4 hours after administration of αMPT versus percent inhibition of brain ChE caused by parathion treatment. Values at 0% inhibition of brain ChE were from rats given only αMPT

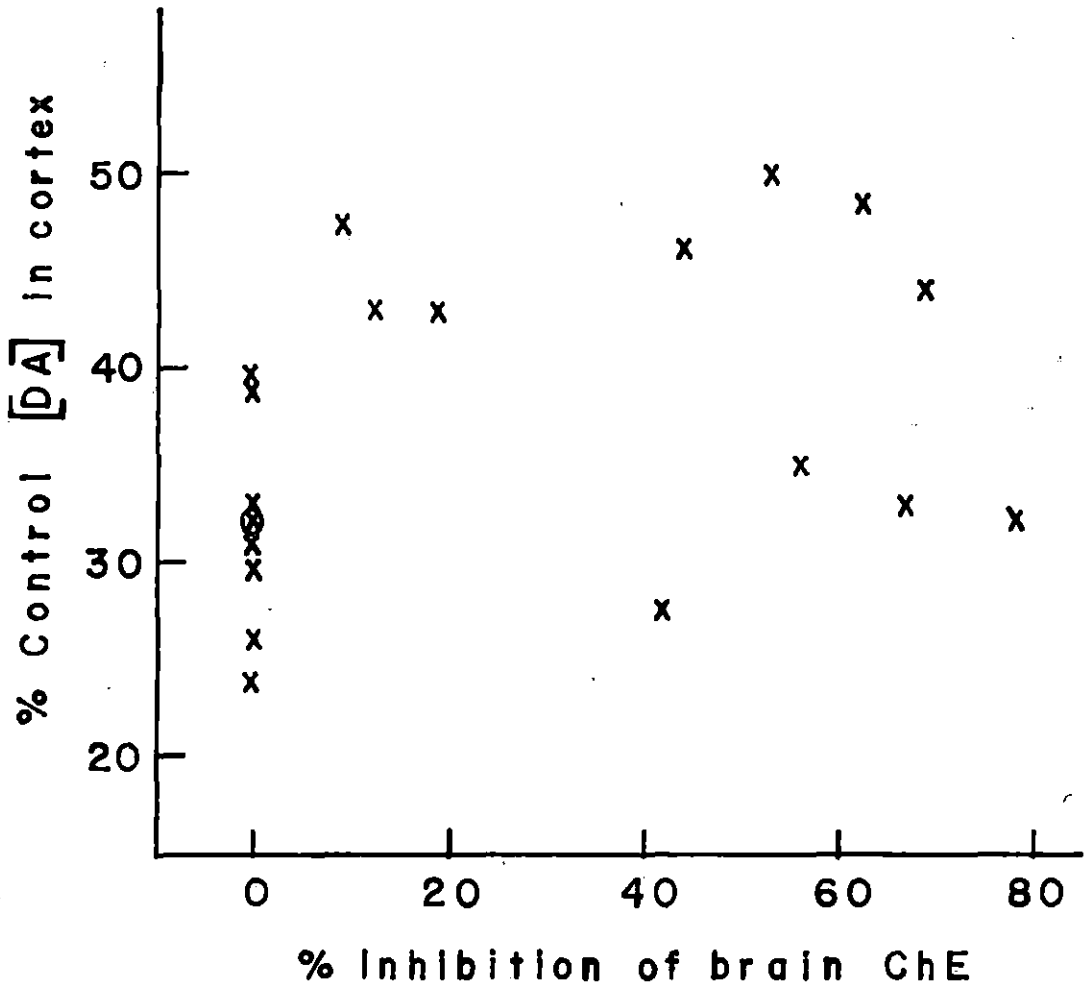

Figure 6. Graph of percent control [DA] in rat cortex 4 hours after administration of αMPT versus percent inhibition of brain ChE caused by parathion treatment. Values at 0% inhibition of brain ChE were from rats given only αMPT

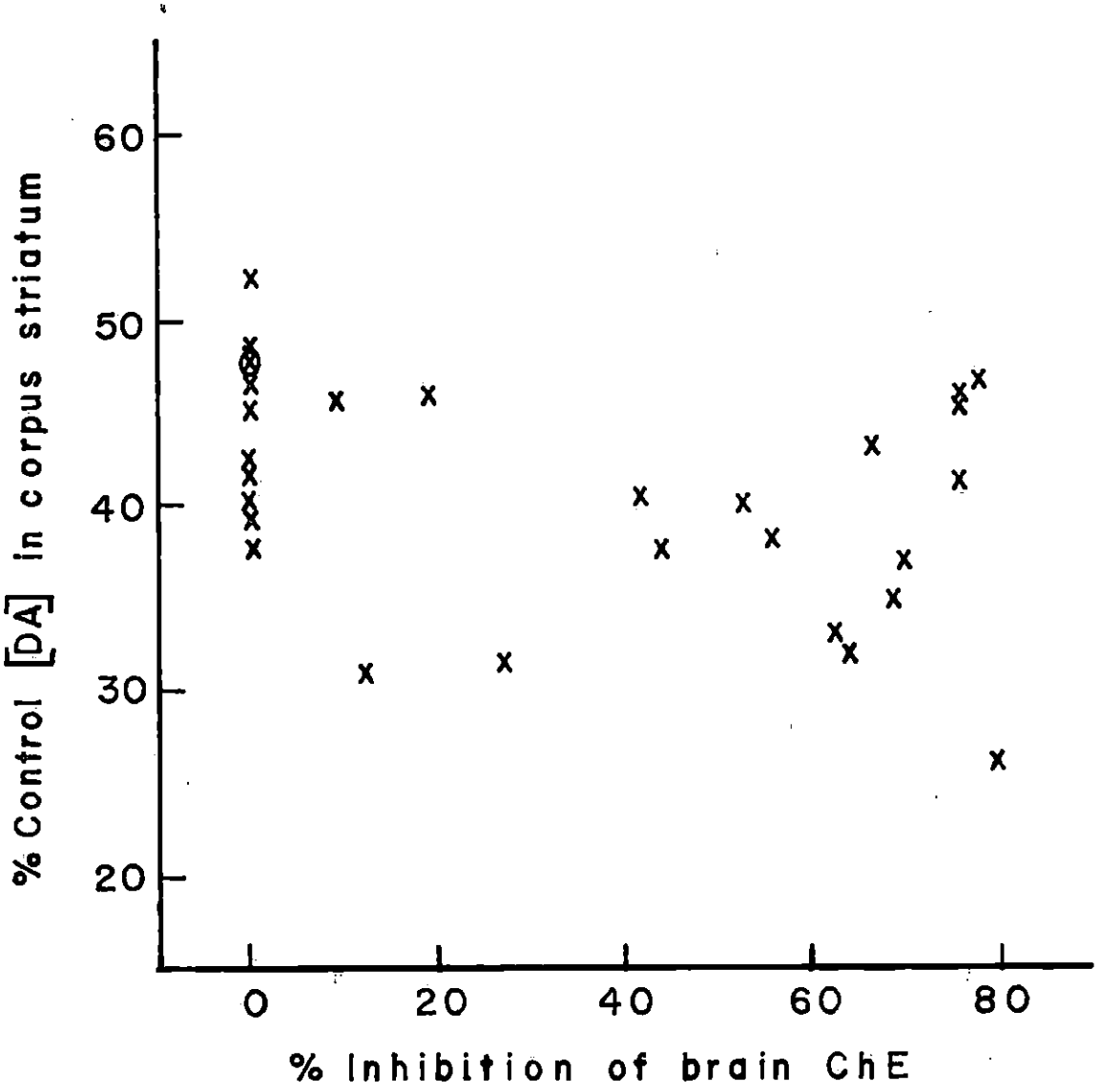

Figure 7. Graph of percent control [DA] in rat corpus striatum 4 hours after administration of αMPT versus percent inhibition of brain ChE caused by parathion treatment. Values at 0% inhibition of brain ChE were from rats given only αMPT

DISCUSSION

The enzymes involved in the synthesis of DA and NE in the mammalian CNS are tyrosine hydroxylase, aromatic L-amino acid decarboxylase, and dopamine-β-hydroxylase (cf. Figure 8). Tyrosine hydroxylase, which catalyzes the hydroxylation of tyrosine, is considered the rate-limiting step in CA synthesis (Nagatsu et al., 1964; Udenfriend, 1966). Two mechanisms of endogenous control of tyrosine hydroxylase activity are: (1) end-product inhibition, in which the catechol products compete with the pteridine cofactor (Udenfriend et al., 1965; Costa and Meek, 1974) and (2) induction of tyrosine hydroxylase synthesis (Musacchio et al., 1969). The endogenous control of synthesis maintains relatively constant levels of NE and DA in the face of varying rates of NE and DA utilization. Therefore, steady-state levels of NE and DA are maintained. The steady-state levels of a neurotransmitter can be defined as that which exists when the rate of synthesis of the neurotransmitter is balanced by its rate of catabolism (or efflux) (Costa, 1970).

Ideally, turnover rate of a neurotransmitter measures the utilization rate of transmitter and reflects the rate of synaptic transaction (Costa and Meek, 1974). The steady-state level of a neurotransmitter, on the other hand, measures the amount of transmitter in the tissue, most of which is stored within the neurons. The amount of transmitter that actually reaches the receptor is presently impossible to measure (Costa and Meek, 1974) and probably is extremely small.

Turnover rates of NE and DA have been determined in various regions of the mammalian brain (Brodie et al., 1966; Iversen and Glowinski, 1966;

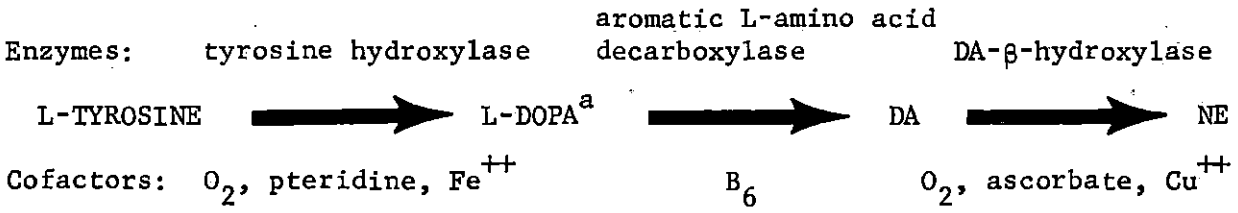

Figure 8. Biosynthesis of DA and NE in the CNS. Tyrosine, which is permeable to the blood-brain barrier, is supplied to the brain from the blood (adapted from Costa and Neff, 1970)

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[a]L-DOPA = L-3,4- dihydroxyphenylalanine.

Costa and Neff, 1970). In the present study, two experimental designs were used to determine turnover of DA and NE in rat brain. Both designs utilized the depletion of NE and DA after the administration of αMPT. The first experimental design (Design 1) was essentially that of Brodie et al. (1966), in which CA levels were measured at 0, 2, 4, 6, and 8 hours after the administration of αMPT (see Appendix C). The rate constant of CA efflux was determined from a least squares linear regression of log [CA] versus time. The linear regression of log [CA] versus time was found to be significant (cf. Figure 1) for NE in the hypothalamus ($P<0.005$), NE in the brain stem ($P<0.001$), NE in the cortex ($P<0.005$), and DA in the corpus striatum ($P<0.001$). (The amount of DA data for the cortex was insufficient to test for significance of linear regression.)

The values found in this study for the rate constants and turnover rates of NE in the brain of the adult male hooded rat (cf. Table 1) were essentially in agreement with the values reported by Brodie et al. (1966). They calculated the rate constant and turnover rate of NE in the whole brain of the NIH Sprague-Dawley rat to be 0.17 hr^{-1} and 71 ng/g/hr. In addition, they calculated the rate constant and turnover rate of NE in the whole brain of the Marland Farms Sprague-Dawley rat to be 0.12 hr^{-1} and 36 ng/g/hr.

Brodie et al. (1966) found almost identical turnover times of NE in the rabbit hypothalamus and midbrain (5.3 and 5.1 hours, respectively). They interpreted their data to indicate that each neuron forms NE at a similar rate and that the rate of synthesis in each part of the brain might be a function of the density of the catecholaminergic neurons.

The data presented in the present study, however, showed a significant difference between the rate constants (and, therefore, between turnover times) of NE in different regions of the brain of the adult male hooded rat (cf. Table 1 and Figure 1). This suggests that individual neuronal synthesis rates of NE are not the same for different regions of the rat brain.

The calculated rate constants of DA efflux in the cortex and corpus striatum of the adult male hooded rat (cf. Table 2) were also in agreement with those calculated by Brodie et al. (1966) for the rate constant of DA in the whole brain of the NIH and Marland Farms Sprague-Dawley rats ($0.37\ hr^{-1}$ and $0.28\ hr^{-1}$, respectively). It is of interest to note that the turnover rate of DA in the corpus striatum of the hooded rat using Design 1 (cf. Table 2) was almost identical to the turnover rate of DA calculated by Brodie et al. (1966) for the rabbit caudate nucleus (2800 ng/g/hr). Since the caudate nucleus is a major part of the corpus striatum, the data suggest that the turnover rate of DA in this brain region is not unique to one species.

The second experimental design (Design 2) was similar to that used by Andén et al. (1969b), in which CA levels were measured at 0 and 4 hours after administration of αMPT (see Appendix C). Turnover rate was estimated from the slope of a line connecting the mean log [CA] for the two treatments (0 and 4 hour groups). The linear regression of log [CA] versus time was found to be significant using Design 1 (see above).

The values calculated for the rate constants and turnover times of NE and DA using Design 2 were in agreement with the values obtained using Design 1 (cf. Tables 3 and 4). This would be expected, since the method of determination of CA turnover is essentially the same in each experimental

design. (Both designs utilize the rate of CA depletion after synthesis inhibition with αMPT.)

A larger deviation was noted, however, in comparing turnover rates calculated from the two experimental designs (especially of DA in the corpus striatum). This deviation was due to the differences in the measured steady-state levels of NE and DA. Variations in NE and DA levels were noted from day to day and between groups of animals.

The data indicated that the measurement of rate constant (k) of CA was a more consistent measurement than turnover rate (K). Also, the rate constant was independent of CA steady-state levels and of the density of CA-containing neurons within the tissue analyzed. For these reasons, the rate constant was selected for use as the index of turnover of NE and DA in brain tissue.

The data showed that both experimental designs gave essentially the same values for the rate constants of NE and DA. Since the primary purpose of this study was to test for the effects of parathion treatment on CA turnover, Design 2 was chosen in preference to Design 1 for the second part of this study. Additional considerations were that Design 2 required fewer experimental units to give the same number of degrees of freedom in the statistical testing of significant differences of the treatment effects. In Design 2, no degrees of freedom are used to test linearity of log [CA] depletion versus time. As stated above, the significance of linearity of log [CA] versus time after αMPT was determined using Design 1. In addition, the amount of time required to carry out an experiment using Design 2 was considerably less than that for Design 1. This made it easier to complete an experiment in one day. Also, all parathion-treated animals were

sacrificed 4 hours after parathion treatment. Therefore, less error was introduced in Design 2 with regard to the drug contact time as compared with Design 1.

Many findings have indicated an interaction of cholinomimetic and cholinolytic drugs with catecholaminergic neurons in the CNS. Cholinomimetic and cholinolytic drugs, for the most part, have had a minimal effect on the steady-state levels of NE and DA in the CNS but a marked effect on turnover. For example, Andén and Bédard (1971) as well as Bowers and Roth (1972) found that antimuscarinic (atropine-like) drugs did not significantly change endogenous (steady-state) levels of DA and NE in rat brain. Pscheidt et al. (1966) found that physostigmine in convulsive and nearly lethal doses had only minimal effects on brain concentration of NE in rabbits and rats. However, a small but significant decrease in the level of NE in the rat brain stem was found at 30 minutes and 4 hours after administration of physostigmine (3 mg/kg, i.p.). They suggested that the depletion of NE in rat brain stem found after treatment with physostigmine may be ascribed to the effects of the convulsive or nonspecific stress-induced release of NE.

However, Glisson et al. (1972, 1974) observed that DFP, an irreversible anti-ChE, caused a decrease in NE levels and a marked elevation of DA levels in rabbit brain as measured 1 hour after administration of DFP. Both responses were blocked by atropine, but only the DA increase was blocked by atropine methylnitrate. Atropine methylnitrate is a muscarinic blocking drug, which does not easily pass the blood-brain barrier unless unusually high doses are employed over a long period of time. They suggested that

the decrease in the NE levels was central in origin, while the elevation of DA levels was peripheral.

In the present study, parathion (1.25 - 2.50 mg/kg) was given i.p. to adult male hooded rats. The dose of parathion was adjusted between 1.25 and 2.50 mg/kg from day to day to obtain a dose that would be sublethal, yet give some classical observable toxic signs. From this experiment and from preliminary experiments, observable toxic signs did not become apparent until brain ChE was inhibited greater than approximately 50%. The symptomatology in order of appearance is: muscular weakness, ataxia, muscular twitching, fasciculations, tremor, compulsive gnawing, and convulsions. The first signs correspond to approximately 50% inhibition of brain ChE and the convulsions with approximately 80% inhibition of brain ChE. In other words, the degree of severity appeared to follow the degree of brain ChE inhibition. Since atropine methylnitrate was given to all treated rats prior to administration of parathion, usual peripheral toxic signs of muscarinic origin, such as salivation, lacrimation, urination, and defecation, were antagonized.

Parathion was found to have no significant effects on steady-state levels of NE and DA in the selected brain regions (cortex, brain stem, and corpus striatum), except for NE in the cortex, where a small but significant decrease in the level of NE was observed (cf. Table 5). Also, parathion treatment, which caused greater than 50% inhibition of brain ChE, gave the same results as above (cf. Table 6).

Since atropine methylnitrate was given to all treated rats, peripheral muscarinic effects of parathion were antagonized. This may explain the discrepancy between the marked elevation of DA levels noted by Glisson

et al. (1972, 1974) after DFP treatment and its absence in the present study in which parathion was used as the anti-ChE. Also, the decrease in NE levels in the cortex (apparently via a central mechanism) observed in this experiment is similar to the results observed by Glisson et al. (1974), in which the NE levels in the thalamus, hypothalamus, midbrain, and hippocampus of the rabbit were lowered by DFP.

Antimuscarinic drugs have been found to decrease the turnover of DA in the adult hooded rat (Andén and Bédard, 1971), in the male Sprague-Dawley rat (Bowers and Roth, 1972), and in the female Wistar rat (Bhatnagar, 1973, 1974). However, Bartholini and Pletscher (1971) suggested that the effect of atropine on DA turnover in the CNS may depend on the route of administration and demonstrated that atropine administered i.p. to male Wistar rats causes a decrease in the HVA level in the brain, while intraventricular administration of atropine causes an increase in brain HVA. In the above study, the level of HVA was used as an index of brain DA turnover, i.e. an increase in brain HVA levels corresponded to an increase in DA turnover. They suggested two cholinergic systems in rat brain, one which causes an increase in DA turnover and another which causes a decrease. The response of a particular cholinergic system depends on the route of administration, i.e. atropine given i.p. causes a decrease in DA turnover, while atropine given intraventricularly causes an increase.

Bhatnagar (1973, 1974) found that atropine and hemicholinium-3 (HC-3), an inhibitor of choline uptake (and consequently an inhibitor of ACh synthesis), causes a decrease in DA turnover in rat brain, while physostigmine, which prolongs the action of ACh, causes an increase in DA turnover. He observed opposite effects of these drugs on NE turnover, i.e. atropine

and HC-3 caused an increase in NE turnover in rat brain, while physostigmine caused a decrease in NE turnover. The above drugs were given intraperitoneally. It is of interest that HC-3 had an effect on CA's in the CNS. HC-3 is a quaternary amine and should not pass the blood-brain barrier (Bowman et al., 1968). It is possible that HC-3 was acting via a peripheral mechanism, which indirectly affected the CNS as with atropine methylnitrate (see above). Another possibility is that the blood-brain barrier may have been altered, thus allowing some HC-3 to enter the brain.

Atropine and HC-3 were also found to inhibit the CPZ-induced increase in DA turnover in rat brain but enhance the CPZ-induced increase in NE turnover. Physostigmine produced the opposite effects in each case (Bhatnagar, 1974). Likewise, Andén and Bédard (1971), Andén (1972), and Bowers and Roth (1972) found that antimuscarinic drugs block both the CPZ- and haloperidol-induced increase in DA turnover. Further, antimuscarinic drugs were found to cause an enhancement of the haloperidol-induced increase in NE turnover (Andén and Bédard, 1971). This suggests the involvement of a cholinergic (muscarinic) mechanism in the CPZ- or haloperidol-induced increase in NE and DA turnover in the CNS.

In the present study, a significant increase in the amount of depletion of DA 4 hours after administration of αMPT was observed in the corpus striatum of the adult male hooded rat as a result of parathion treatment (cf. Table 8 and Figure 3). This parathion-induced increase in the depletion of DA in the rat corpus striatum is an indication of an increase in the turnover of DA in that brain region. The effect of parathion on DA depletion after αMPT did not appear to be dependent upon the percent inhibition of brain ChE (cf. Figure 7). This suggests that parathion may be

acting on the dopaminergic system in the corpus striatum via a mechanism independent of its anti-ChE property.

In contrast, a decrease in the amount of depletion of DA after αMPT administration was observed in the rat cortex as a result of parathion treatment (cf. Table 8 and Figure 3). A parathion-induced decrease in DA turnover in the rat cortex is indicated. The effect of parathion on DA depletion after αMPT did not appear to be dependent upon the percent inhibition of brain ChE (cf. Figure 6). This suggests that parathion may be acting on the dopaminergic system in the cortex via a mechanism independent of its anti-ChE property.

The amount of depletion of NE in the rat brain stem after αMPT administration was significantly increased by parathion treatment which had caused greater than 60% inhibition of brain ChE (cf. Table 7 and Figure 2). A parathion-induced increase in the turnover of NE in the rat brain stem is indicated. The effect of parathion on NE depletion in the brain stem after αMPT appeared to be dependent upon the percent inhibition of brain ChE (cf. Figure 4). This suggests that parathion may be acting on the noradrenergic system in the brain stem via a mechanism of ChE inhibition.

Parathion did not significantly alter the amount of depletion of NE in the cortex of the adult male hooded rat after αMPT administration (cf. Table 7 and Figure 2). However, the effect of parathion on the depletion of NE in the rat cortex appeared to be dependent upon the percent inhibition of brain ChE (cf. Figure 5). This suggests that parathion may be acting on the noradrenergic system in the cortex via a mechanism of ChE inhibition. Based on the data presented in Figure 5, a significant increase in the amount of NE depletion in the rat cortex after αMPT may have been

obtained if more data at the higher percent inhibition (60 to 80%) of brain ChE had been accumulated.

The interpretation of the data for the effect of parathion on the depletion of NE in the rat cortex is further complicated by the fact that parathion caused a significant decrease in the steady-state level of NE in the cortex (cf. Tables 5 and 6). When Design 2 is used to determine the effects of a drug on the turnover of NE or DA, the drug should not affect the steady-state levels of NE or DA. Otherwise, the levels of NE and DA measured 4 hours after administration of αMPT may also be affected, possibly by a mechanism unrelated to the turnover.

Many factors are involved in the turnover of NE and DA, such as the rate of release of NE and DA from the nerve terminal, the rate of reuptake at the presynaptic membrane, the rate of metabolism, etc. Drugs affecting one or many of these factors may influence the turnover of DA and NE. Parathion may be acting directly on any of these factors or may be acting via its anti-ChE property.

The mechanism of parathion's action as an inhibitor of monoamine oxidase (MAO) or catecholamine-O-methyltransferase (COMT) could explain some of its effects on CA turnover. However, inhibition of MAO or COMT should have affected the steady-state levels of NE and DA, i.e. caused an elevation of CA levels, especially DA (Kopin, 1972). This was not observed in the present study (cf. Tables 5 and 6). However, the possibility remains that parathion may be affecting other enzymes in the CNS. This in turn may alter the turnover of CA's in the CNS.

Another possibility is that parathion may be altering the reuptake of CA's at the synapse. As previously stated, many anticholinergics have been

shown to inhibit the reuptake of CA's at the synaptic membrane (Coyle and Snyder, 1969). Parathion may be acting via a mechanism similar to that of the anticholinergics.

Also, it is possible that parathion may cause an increased cholinergic stimulation, either directly or indirectly, at the catecholaminergic synaptic membrane. If the cholinergic link hypothesis (Burn and Rand, 1965) is correct, an enhancement of the chemical stimulation of excitatory cholinergic (nicotinic) receptors at the synaptic membrane may increase the release and, therefore, the turnover of CA's in the CNS.

In addition, Löffelholz and Muscholl (1969) have suggested the presence of inhibitory cholinergic (muscarinic) receptors at the sympathetic nerve terminals in the heart. They found that muscarinic stimulants caused a decrease in the release of NE from electrically stimulated sympathetic nerves, while atropine caused an increase. If such inhibitory muscarinic receptors are present at the synaptic membrane of catecholaminergic neurons in the CNS, enhancement of cholinergic stimulation by parathion may cause a decrease in the release and, therefore, the turnover of CA's in the CNS.

The relative population of muscarinic-inhibitory and nicotinic-excitatory receptors at the synaptic membrane of catecholaminergic neurons in various regions of the CNS may determine whether the turnover of CA's is decreased or increased during enhancement of endogenous cholinergic stimulation. It is possible that the muscarinic-inhibitory receptors predominate at the synaptic membrane of dopaminergic neurons in the cortex, causing a decrease in the DA turnover during enhancement of cholinergic stimulation. Further, the nicotinic-excitatory receptors may predominate at the synaptic membrane of dopaminergic neurons in the corpus striatum,

causing an increase in DA turnover in that brain region during enhancement of cholinergic stimulation.

Another possible explanation of parathion's effects on CA turnover is the presence of cholinergic receptors in the CNS that affect the impulse activity of catecholaminergic neurons. Two models can be hypothesized: (1) a direct synapse of cholinergic neurons on catecholaminergic neurons in the CNS and (2) cholinergic neurons which synapse with a chain of "interneurons" ultimately affecting the impulse activity of the catecholaminergic neurons in the CNS. Again, this may explain the difference in the effects of parathion on the turnover of DA in the two brain regions studied. Cholinergic input to dopaminergic neurons may be predominantly inhibitory in the cortex but predominantly excitatory in the corpus striatum.

Findings that suggest the presence of both catecholaminergic and cholinergic neurons in the mammalian CNS give support to the above hypothesized models. Indeed, Shute and Lewis (1966) have described the presence of a high density of cholinergic and dopaminergic neurons in the corpus striatum. They defined cholinergic neurons as those whose cell bodies stained heavily for AChE and gave rise to axons containing AChE. The presence of catecholaminergic neurons in various regions of the CNS was based on the data presented by Dahlström and Fuxe (1964). An anatomical interrelationship of cholinergic and catecholaminergic neurons in the corpus striatum, as well as in other regions of the CNS, seems to be a valid possibility.

In summary, parathion was found to have minimal effects on the steady-state levels of NE and DA in rat brain. However, a small but significant decrease in the level of NE in the cortex was elicited by parathion treat-

ment. Also, parathion was found to cause an increase in DA turnover in the rat corpus striatum but a decrease in DA turnover in the rat cortex. The effects of parathion on DA turnover did not appear to be dependent upon the percent inhibition of brain ChE. Further, parathion caused an increase in NE turnover in the rat brain stem, an increase that appeared to be dependent upon the percent inhibition of brain ChE.

SUMMARY

The primary objective of the present study was to determine the effects of parathion on the steady-state levels and turnover of NE and DA in selected regions of rat brain. An interdependence of the catecholaminergic and cholinergic systems in the mammalian brain was proposed as a working hypothesis. Parathion was employed as a pharmacological agent to enhance the endogenous cholinergic stimulation in the CNS. Two parameters (steady-state levels and turnover) of brain NE and DA were measured to determine effects of parathion treatment on the catecholaminergic system in the mammalian brain.

Prior to the study, preliminary experiments were undertaken to determine an appropriate experimental design to test treatment effects on CA turnover. The values obtained using two experimental designs were compared. In the first experimental design (Design 1), levels of NE and DA were measured at 0, 2, 4, 6, and 8 hours after synthesis inhibition with αMPT. The linearity of the plot of log[CA] versus time was found to be significant. Steady-state levels, rate constants, turnover times, and turnover rates of CA's were determined in the selected rat brain regions (cerebral cortex, brain stem, hypothalamus, and corpus striatum). Significant differences were found between the rate constant of NE in the cortex and the rate constants of NE in the brain stem and hypothalamus. Likewise, a significant difference was found between the rate constant of NE in the brain stem and the rate constant of NE in the hypothalamus. However, no significant difference was found between the rate constants of DA in the cortex and the corpus striatum.

In the second experimental design (Design 2), levels of NE and DA were determined at 0 and 4 hours after synthesis inhibition with αMPT. No degrees of freedom were used to statistically determine the linearity of log[CA] depletion versus time. Therefore, more degrees of freedom were available as compared to Design 1 for testing significant differences of treatment effects on CA depletion after αMPT (assuming the same number of experimental units in the two experimental designs).

Both experimental designs gave essentially the same values for the rate constants (fractional turnover rates) of NE and DA in the selected rat brain regions. However, a discrepancy was observed between the values of turnover rates determined from the two experimental designs. This discrepancy was attributed to the marked variation in the values of steady-state levels of NE and DA measured on different days. The rate constant was chosen as the index of CA turnover, since it is independent of CA steady-state levels. For the above reasons, Design 2 was chosen in preference to Design 1 for the determination of significant effects of drug treatment on the turnover of NE and DA in brain tissue. More efficient use of the biological material could be made with Design 2 as compared to Design 1.

Parathion was found to have minimal effects on the steady-state levels of NE and DA in rat brain. The effects of parathion on steady-state levels of NE and DA in the selected brain regions were found to be not significant, except for NE in the cortex, where a small but significant decrease in the level of NE was observed after parathion treatment.

Using Design 2 as the experimental design, parathion was found to increase the turnover of DA in the corpus striatum but decreased the turnover of DA in the cortex. The effects of parathion on DA turnover in these

two brain regions appeared to be independent of the percent inhibition of brain ChE. Further, parathion was found to increase the turnover of NE in the brain stem. Also, parathion appeared to increase the turnover of NE in the cortex as well, but the increase was not statistically significant. The effects of parathion on NE turnover in the brain stem and cortex appeared to be dependent upon the percent inhibition of brain ChE.

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APPENDIX A.
REVIEWS OF SPECIFIC AREAS OF CA RESEARCH

1. Biochemistry - Molinoff and Axelrod, 1971.
2. Synthesis - Kopin, 1968; Axelrod, 1971; Costa and Meek, 1974.
3. Uptake - Iversen, 1970, 1971.
4. Transport and storage - Glowinski, 1970; Shore, 1972.
5. Release - von Euler *et al.*, 1966; Glowinski, 1970; Smith and Winkler, 1972.
6. Receptor - Curtis and Crawford, 1969; Triggle, 1972.
7. Metabolism - Axelrod, 1966, 1971; Glowinski and Baldessarini, 1966; Kopin, 1972.
8. Function - Hornykiewicz, 1966; Marley and Stephenson, 1972.
9. Turnover - Costa, 1970; Costa and Neff, 1970.
10. Pharmacology - Salmoiraghi *et al.*, 1965; Sulser and Sanders-Bush, 1971.

APPENDIX B.
FLUOROMETRIC DETERMINATION OF NE AND DA LEVELS IN BRAIN
(cf. Shellenberger and Gordon, 1971)

Reagents:

1. 0.4N Perchloric acid, 0.1% sodium metabisulfate ($Na_2S_2O_3$), 0.05% disodium EDTA.
2. Tricine buffer, 0.1M tricine (N-tris(hydroxymethyl)methylglycine), 0.0525N NaOH, 2.5% disodium EDTA.
3. 0.05N Perchloric acid.
4. Phosphate buffer, 0.1M, pH 7.0, 0.9% disodium EDTA. (Dissolve 4.27 g Na_2HPO_4, 9.52 g KH_2PO_4, and 9.0 g disodium EDTA in approximately 800 ml d.d. H_2O. Adjust pH to 7.0 with 5N NaOH and bring final volume to 1 liter with d.d. H_2O.)
5. Iodine reagent. (Dissolve 2.5 g KI and 1.25 g I_2 in d.d. H_2O and bring to final volume of 50 ml.)
6. Alkaline sodium sulfite solution. (Dissolve 0.500 g $NaSO_3$ in 2.00 ml d.d. H_2O. Add 18.0 ml 5N NaOH. (Note: This solution should always be made up just prior to use.)
7. Alumina. Preparation procedure: Wash a 250 g portion of alumina (Woelm, activity grade 1, Al_2O_3) for 6 to 8 hours with free flowing tap water to remove the lighter particles. Acid-wash the alumina in 1000 ml 2N HCl for 1 hour at 100° C. Keep the alumina suspended with constant stirring. After the first wash, allow the alumina to settle for 1 or 2 minutes before pouring off the acid and lighter particles. Wash the alumina twice in 500 ml 2N HCl at 70° C for 1 hour (again with constant stirring). Pour off the

acid and lighter particles as before. Wash with 1000 ml 2N HCl at 50° C for 1 hour and pour off acid and lighter particles. Bring the pH of the alumina to 3.4 with 25 to 35 washes of 400 ml d.d. H_2O. Transfer the alumina to a porcelain evaporating dish covered with a watch glass and heat in a muffle furnace for 1 hour at 120° C and then for 2 hours at 300° C. Store the final activated alumina in a vacuum desiccator at room temperature.

Standards

1. Stock solutions.

   Add 5.00 ml 0.1N HCl to a NE reference standard.[1] Also add approximately 1 mg of ascorbic acid as an antioxidant.

   Resulting NE solution = 1.00 mg/ml.

   Store this NE stock solution at 4° C. (The NE stock solution will remain good for at least 1 month under these conditions.)

   Dissolve 1.00 mg DA[2] in 1.00 ml 0.1N HCl.

   Resulting DA solution = 1.00 mg/ml.

   (Make up DA stock solution fresh for each assay.)

2. Daily stock solution.

   Bring to volume 0.25 ml of the stock solution of NE and DA with 0.05N $HClO_4$ in a 25 ml actinic glass volumetric flask.

   This gives the following daily stock solutions:

   NE = 0.0100 mg/ml and DA = 0.0100 mg/ml

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[1] Regis Chemical Co. (Code #190012).

[2] 3-Hydroxytyramine (Calbiochem).

3. Recovery standard.

   Add 0.1 ml NE daily stock solution and 0.1 ml DA daily stock solution to 5.8 ml 0.4N $HClO_4$. Prepare recovery blanks by adding 0.2 ml of the same 0.05N $HClO_4$ solution used to prepare the daily stock solutions to 5.8 ml 0.4N $HClO_4$. Treat the recovery standards and recovery blanks in the same manner as the brain samples starting at the first step of the isolation of CA procedure.

4. Standards.

   Add 0.1 ml NE daily stock solution and 0.1 ml DA daily stock solution to 0.8 ml 0.05N $HClO_4$. Prepare standard blanks by adding 0.2 ml of the same 0.05N $HClO_4$ solution used to prepare the daily stock solutions to 0.8 ml 0.05N $HClO_4$. Treat the standards and standard blanks in the same manner as brain samples starting at the first step of the oxidation procedure.

Apparatus:

1. Sonifier Cell Disrupter, Heat Systems Co., with micro tip.
2. Aminco-Bowman Spectrophotofluorometer, American Instrument Co., Inc.
3. SORVALL RC2-B, Refrigerated Centrifuge, Ivan Sorvall Inc.

Procedure:

A. Extraction

1. Remove brain samples as rapidly as possible and store in liquid nitrogen until assayed. Begin extraction as soon as practical (usually within 1 or 2 days).

2. Weigh samples (50 mg to 1 g) of rat brain to the nearest mg and homogenize by ultrasonic cell disruption in 0.4N $HClO_4$ in a 50 ml polypropylene cfg. tube.
3. Allow the tissue homogenate to stand in ice for 10 minutes.
4. Centrifuge at 27,000 g for 15 minutes at 0° C.
5. Transfer the supernatant to a 15 ml polycarbonate, graduated cfg. tube.
6. Add 2.5 ml 0.4N $HClO_4$ to the pellet and rehomogenize.
7. Again let the homogenate stand for 10 minutes.
8. Centrifuge the homogenate as in step 3 above.
9. Pool the two supernatants in the 15 ml polycarbonate cfg. tube and bring to a final volume of 6 ml with 0.4N $HClO_4$.
10. Store samples at -20° C in a freezer until analysis for NE and DA. (Samples may be stored at this step of the extraction procedure for up to 3 weeks at -20° C without apparent loss of NE and DA (Shellenberger and Gordon, 1971).)

B. Isolation of Catecholamines

1. Thaw the 0.4N $HClO_4$ tissue extracts and transfer to 50 ml polycarbonate cfg. tube (Oak Ridge type). Add recovery standards and recovery blanks at this step and treat as brain samples throughout the rest of the procedure.
2. Buffer the extract to pH 7.5 to 8.0 with Tricine buffer. (Check with a pH meter.)
3. Immediately add 300 mg $\pm$ 25 mg of alumina. (CA's are unstable at neutral and alkaline pH.)

4. Tightly cap the cfg. tubes containing the samples and shake at low speed (180/min) for 20 minutes.
5. Centrifuge at 3000 g for 5 minutes.
6. Discard the supernatant and wash the alumina 4 times with approximately 30 ml d.d. H_2O. Centrifuge the last wash at 3000 g for 5 minutes and remove the water by aspiration.
7. Add 3.0 ml 0.05N $HClO_4$ to each alumina sample, cap the cfg. tube, and shake for 20 minutes at low speed.
8. Centrifuge at 3000 g for 5 minutes.
9. Transfer a 1.00 ml aliquot of the supernatant to a 13x100 mm culture tube for oxidation.

C. Oxidation to Fluorescent Product and Estimation of NE and DA Levels

1. Buffer each 1 ml aliquot of the 0.05N $HClO_4$ eluate with 1.5 ml 0.1M phosphate buffer. (The pH of each sample should be 6.5 $\pm$ 0.2.) Add the standards and standard blanks at this step and treat as brain samples throughout the rest of the procedure.
2. Add 0.2 ml of iodine reagent to each of the samples followed exactly 2.00 minutes later by 0.5 ml of alkaline sodium sulfite solution. Exactly 2.00 minutes later add 0.4 ml of glacial acetic acid. Mix thoroughly after each addition. (See Notes for the chemical reactions involved at this step.)
3. Heat the samples in a temperature block at 100° C for 3 to 4 minutes.
4. Cool in ice for 3 to 5 minutes.
5. Allow samples to stand at room temperature for 15 minutes.

6. Transfer samples to glass SPF cuvettes (10x75 o.d. round cell[1]) for measurement of NE fluorescence.
7. Place a standard in the SPF for fluorescence measurement and manually adjust the SPF wavelength disc to peak excitation and emission wavelengths of NE fluorescence. (The usual peak excitation and emission wavelengths of NE fluorescence are approximately 380 nm and 480 nm, respectively.) Use these wavelengths in the measurement of NE fluorescence of the samples.
8. Make all fluorescence measurements in duplicate with the second reading in the reverse order of the first. Take the average of the two readings. (This reading procedure minimizes time-dependent effects on fluorescence.)
9. Return the samples to their respective 13x100 mm culture tube and place them in the temperature block for an additional 35 to 40 minutes.
10. Place the culture tubes in ice for 3 to 5 minutes followed by standing at room temperature for 30 minutes before reading DA fluorescence. (This is a departure from the Shellenberger and Gordon method. See Notes.)
11. Transfer the samples to the SPF glass cuvettes.
12. Adjust the SPF to peak excitation and emission wavelengths for DA fluorescence using the DA standard. (Usual peak wavelengths are 320 nm and 373 nm.)
13. Make duplicate measurements of DA fluorescence as with NE.

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[1] Aminco-Bowman.

14. The calculation of CA (NE and DA) levels in brain samples are as follows:

$$\%Rec = \frac{RF(rec) - RF(recblk)}{RF(std) - RF(stdblk)} \times 3 \times 100$$

$$[CA] = \frac{[RF(sample) - RF(recblk)](1000\ ng/ml)(3\ ml)}{(RF(std) - RF(stdblk))(\%Rec/100)(sample\ weight\ in\ grams)}$$

[CA] is calculated as ng/g of brain tissue (wet weight).

%Rec - percent recovery

RF(rec) - relative fluorescence of the recovery

RF(recblk) - relative fluorescence of the recovery blank

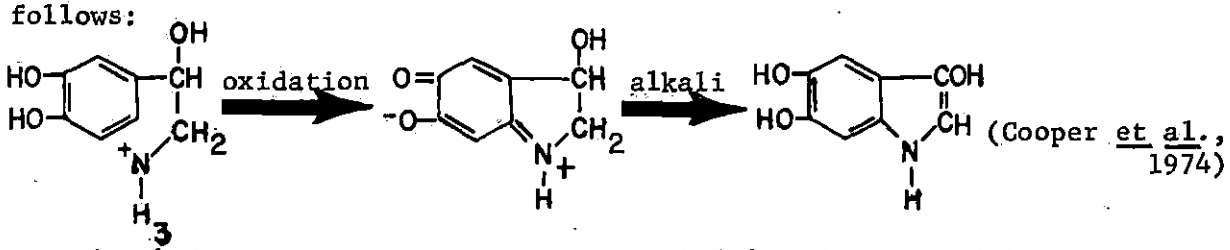
RF(std) - relative fluorescence of the standard

RF(stdblk) - relative fluorescence of the standard blank

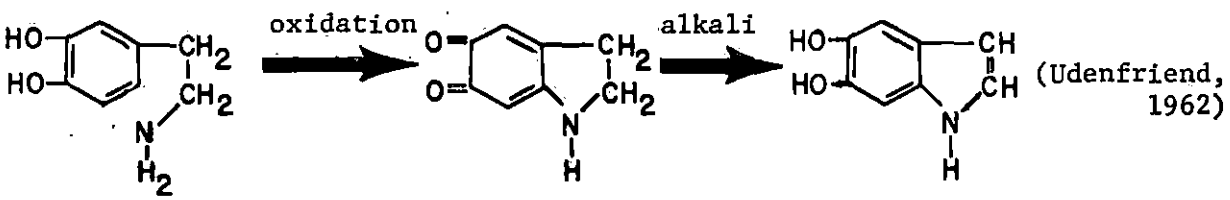
RF(sample) - relative fluorescence of the sample

Notes:

1. The chemical reaction involved in the oxidation procedure is as follows:



norepinephrine — 3,5,6-trihydroxyindole



dopamine — 5,6-dihydroxyindole

3,5,6-trihydroxyindole and 5,6-dihydroxyindole are the highly fluorescence products formed in the oxidation procedure. Each product has a characteristic wavelength of excitation and emission.

2. In the procedure for the estimation of DA levels, the samples were allowed to stand at room temperature for 30 minutes before reading DA fluorescence. This is a departure from the Shellenberger and Gordon method, since their method calls for the reading of DA fluorescence at 4° C. However, problems were encountered while using their method in this laboratory during previous experiments. Because of the laboratory humidity, the cuvettes would fog up during the reading of DA fluorescence and would give erroneously high readings, especially for blanks and samples of low DA concentration.

Shellenberger and Gordon (1971) stated that a stable fluorescence is obtained if the samples are allowed to sit at room temperature for over 30 minutes. However, this stable fluorescence at room temperature is 15 to 20% lower in intensity than that obtained at 4° C. It was found that the increase in reproducibility of readings and the lowering of the blank fluorescence more than compensated for the loss of sensitivity due to the reduction in fluorescence intensity.

APPENDIX C.
DETERMINATION OF BRAIN NE AND DA TURNOVER

The turnover rates of NE and DA in rat brain are determined from the rate of depletion of NE and DA after administration of αMPT (Brodie et al., 1966). αMPT competitively inhibits tyrosine hydroxylase, the enzyme that catalyses the rate-limiting step in the synthesis of CA's (Moore and Dominic, 1971). The NE and DA levels in rat brain are observed to decrease exponentially after injection of αMPT (200 mg/kg, i.p.).

According to Brodie et al. (1966):

$$-d[NE]/dt = k[NE] \tag{1}$$

where [NE] is the concentration of NE at time t and k is the rate constant of NE efflux. The value k can be thought of as the fraction of the total NE that is lost per unit time (i.e. fractional turnover rate).

The turnover rate, K, is described as:

$$K = k[NE]_0 \tag{2}$$

where $[NE]_0$ is the initial concentration of NE (i.e. the steady-state level of NE). The NE in brain tissue is assumed to be at steady-state prior to the administration of αMPT. A steady-state condition exists when the rate of NE efflux from the tissue equals the rate of NE biosynthesis.

Integration of equation (1) gives:

$$[NE] = [NE]_0 e^{-kt} \tag{3}$$

which converted to $\log_{10}$ gives:

$$\log[NE] = \log[NE]_0 - 0.4343kt \tag{4}$$

From equation (4), a straight line with slope = -0.4343k and y-intercept = $\log[NE]_0$ occurs when log[NE] is plotted on the y-axis and time (t)

on the x-axis. Therefore, the rate constant (k) can be calculated from the slope (b):

$$k = -b/0.4343 \quad (5)$$

where b can be determined from a least squares fitting of the linear regression of log[NE] versus t.

The turnover rate (K) can be calculated from equation (2). The turnover time (tt) is the reciprocal of k.

$$tt = 1/k \quad (6)$$

Brodie et al. (1966) defines turnover time of NE as the time interval required for the biosynthesis of an amount of NE equal to that stored in the tissue at steady state.

Rate constants, turnover times, and turnover rates of DA in brain tissue are calculated in a similar manner.

Two experimental designs for the determination of brain NE and DA turnover are used in this study.

DESIGN 1.

Rat brain DA and NE levels are determined at 0, 2, 4, 6, and 8 hours after administration of αMPT (200 mg/kg, i.p.). A second dose of αMPT (100 mg/kg, i.p.) is given 2.5 hours after the first dose to rats in the 4, 6, and 8 hour groups. The second dose of αMPT is given to assure maintenance of effective levels of αMPT in the brain throughout the experiment.

An example of the determination of NE turnover using this experimental design is given. The raw data used for these calculations are reported in Table C-1.

Table C-1. Raw data of [NE] in rat brain stem after administration of αMPT (200 mg/kg, i.p.) using Design 1

t[a] (hr)	[NE][b] (ng/g)	log[NE]
0	409.7	2.612
2	275.5	2.440
4	190.5	2.280
6	141.3	2.150
8	109.1	2.038

[a]Hours after administration of αMPT.

[b]Concentration of NE in rat brain stem at selected time intervals after administration of αMPT. Brain stems were pooled from the 4 rats in each time group.

The graph of log[NE] versus t (cf. Figure C-1) shows log[NE] to decrease linearly with time. A least squares analysis of log[NE] versus t shows the linear regression to be significant at the 0.5% probability level.

CALCULATIONS:

$$\log[NE]_0 = \text{y-intercept} = 2.592$$

$$[NE]_0 = 390.8 \text{ ng/g}$$

$$b = -0.07196$$

$$k = -b/0.4343 = 0.1657 \text{ hr}^{-1}$$

$$tt = 1/k = 6.03$$

$$K = k[NE]_0 = 64.8 \text{ ng/g/hr}$$

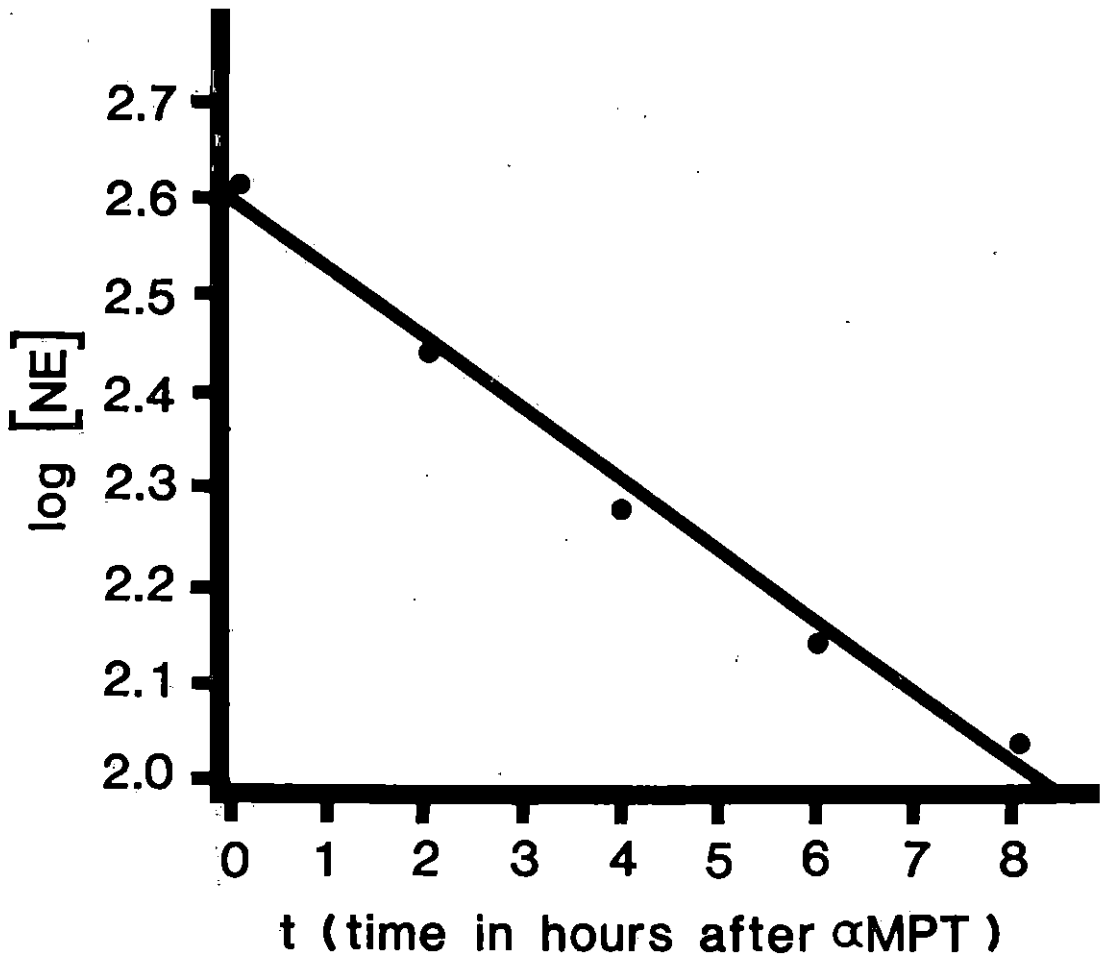


Figure C-1. Graph of log[NE] ([NE] in ng/g) in rat brain stem after administration of αMPT (200 mg/kg, i.p.)

Rats in the 4, 6, and 8 hour groups were given a second dose of αMPT (100 mg/kg, i.p.) 2.5 hours after the first dose. The least squares linear regression of log[NE] versus t was significant ($P<0.005$). Each dot on the graph represents the log[NE] of a pooled sample of 4 rat brain stems.

The rate constants, turnover times, and turnover rates of NE and DA in other brain regions are calculated in a similar manner.

DESIGN 2.

In this experimental design, all treated rats are sacrificed 4 hours after administration of αMPT (200 mg/kg, i.p.). Less biological material is needed with this design as compared to Design 1 for testing significant differences of treatments that affect CA depletion after αMPT. In Design 2, no degrees of freedom are used to determine significance of linearity of log[NE] versus time.

The fixed time interval, 4 hours, is chosen because it is approximately the ½-life of DA and NE depletion in rat brain after αMPT (cf. Table C-2). A second dose of αMPT is not given, because it has been reported that effective levels of αMPT in rat brain are maintained for 4 hours after administration of αMPT (200 mg/kg, i.p.) (Spector et al., 1965; Brodie et al., 1966).

An example of the determination of CA turnover in rat brain using this experimental design is given:

Three or four individual experiments were carried out on different days. In each individual experiment, 2 to 4 adult male hooded rats (200-400 g) were used for the control group and a similar number for the αMPT treated group. Data obtained for the determination of turnover of DA in the rat corpus striatum using Design 2 is given in Table C-3. The graph of log[DA] versus time is given in Figure C-2. The rate constants were calculated from the slope of the line connecting the pooled data means of log[DA] at 0 and 4 hours.

Table C-2. Half-life[a] of DA and NE depletion in rat brain after administration of αMPT (200 mg/kg, i.p.)

	Half-life (hr)	
	DA	NE
Cortex	2.48	3.18
Brain stem	---[b]	4.18
Hypothalamus	---[b]	5.63
Corpus striatum	3.30	---[b]

[a] $t_{\frac{1}{2}} = -(\log 2)/b$; where b = slope of the linear regression of log[CA] vs. time after administration of αMPT. The values calculated for half-life of DA and NE were based on the data obtained using Design 1.

[b] Not determined.

Calculations:

$$b = \frac{\log[DA]_4 - \log[DA]_0}{4}; \quad \log[DA]_0 = 3.962 \pm 0.0274$$

$$\log[DA]_4 = 3.595 \pm 0.0185$$

$$b = -0.09175 \pm 0.0115$$

$$k = -b/0.4343 = 0.2113 \pm 0.0264$$

$$[DA]_0 = 9360 \pm 567$$

$$K = k[DA]_0$$

$$K = (X_1 \pm \sigma_1)(X_2 \pm \sigma_2) \doteq X_1X_2 \pm \sqrt{X_2^2 s_1^2 + X_1^2 s_2^2}$$ [1]

$$X_1 = 9360 \qquad s_1 = 567$$

$$X_2 = 0.2113 \qquad s_2 = 0.0264$$

$$K = 1978 \pm 275$$

[1] An approximation of the standard error. Adapted from Stout (1950).

Table C-3. Raw data of DA in rat corpus striatum after administration of αMPT (200 mg/kg, i.p.) using Design 2

Day[a]	[DA] (ng/g)		log[DA]	
	Control[b]	αMPT[c]	Control	αMPT
1	12120[d]	5209	4.083	3.717
1	8951	4758	3.952	3.677
2	6601	3764	3.820	3.576
2	6670	3392	3.824	3.530
2	7520	3898	3.876	3.591
3	11020	3958	4.042	3.597
3	8933	4037	3.951	3.606
3	7331	2995	3.865	3.476
3	10900	3738	4.037	3.573
4	11560	3624	4.063	3.559
4	10600	3903	4.025	3.591
4	10110	4450	4.005	3.648
Mean	9360	3977	3.962	3.595
$S/\sqrt{n}$	567	172	0.0274	0.0185

[a]The total data were pooled from 4 individual experiments carried out on different days.

[b]Control rats were given an equivalent volume of saline and sacrificed within 1 hour.

[c]αMPT treated rats were sacrificed 4 hours after administration of αMPT (200 mg/kg, i.p.).

[d]Each [DA] value represents the calculated DA concentration of the pooled corpus striatum of one rat.

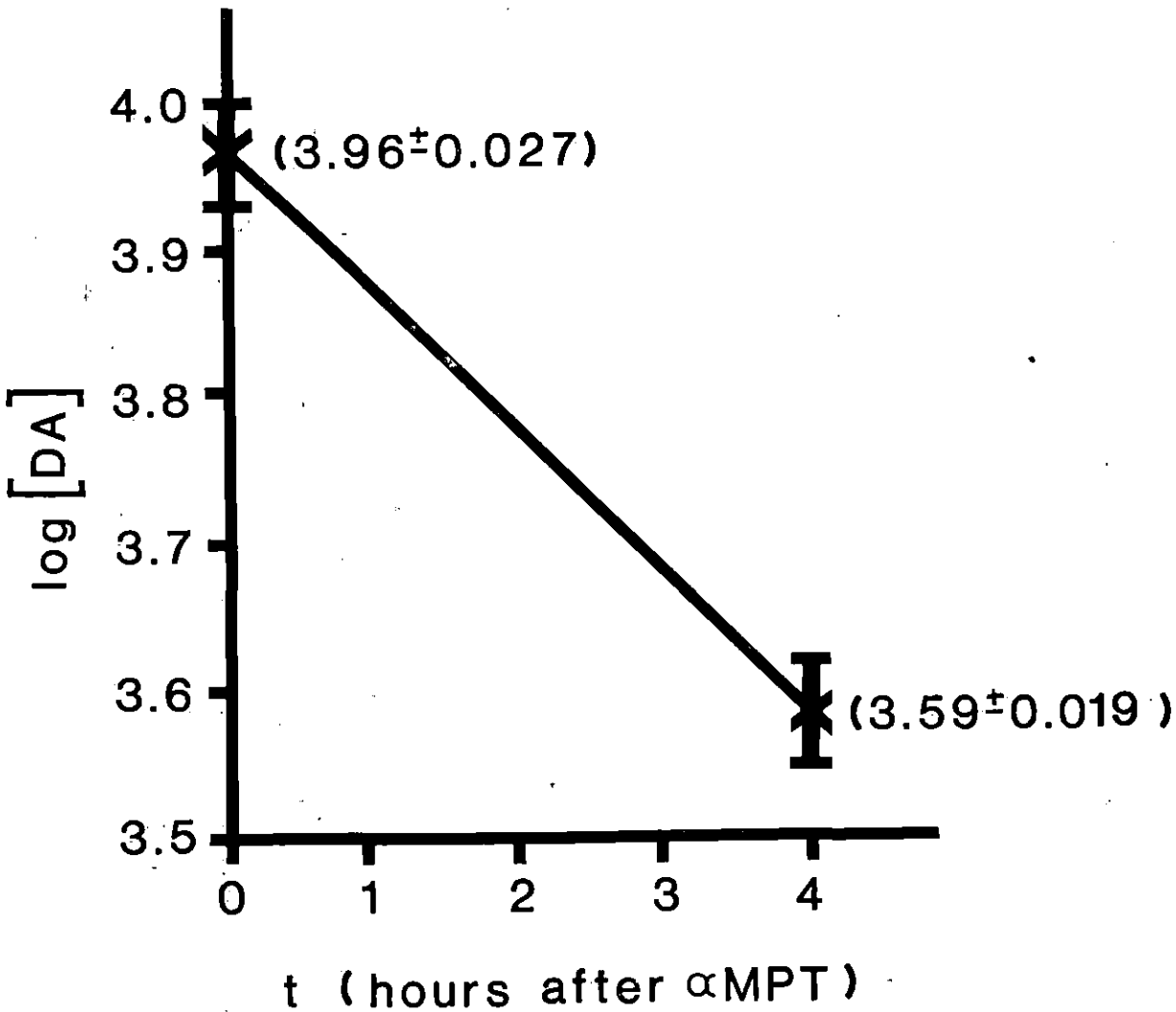


Figure C-2. Graph of mean log[DA] ± the standard error of the mean in rat corpus striatum at 0 and 4 hours after administration of αMPT (200 mg/kg, i.p.). The slope of the line was used to calculate k, the rate constant of DA efflux

APPENDIX D.
DETERMINATION OF ChE ACTIVITY BY pH-STAT METHOD
(cf. Glick, 1971)

Reagents:

1. Medium (0.1M NaCl, 0.02M $CaCl_2$. Add 5.845 g NaCl and 2.220 g $CaCl_2$ to d.d. H_2O and bring to 1 liter volume with d.d. H_2O.)
2. Titrant (0.0200M NaOH, CO_2-free. Dilute a CO_2-free stock solution of 1M NaOH with boiled d.d. H_2O. The exact concentration of the stock solution is checked periodically against potassium hydrogen-phthalate standard. Prepare titrant for each assay.)
3. Substrate (0.250M ACh-Cl. Add 3.3 ml d.d. H_2O to a vial of 150 mg ACh-Cl.[1] Prepare for each assay.)
4. Tissue homogenate (Homogenize tissue in 10 volumes of 0.1M NaCl.)

Apparatus:

Titrator, Radiometer type TTT2b

Titrigraph Pen Drive, Radiometer type REA 300

Servograph, Radiometer type REC 51

Autoburette, Radiometer type ABU11 (0.25 ml burette)

pH Electrodes

Micro-glass electrode, Radiometer #G2222c

Micro-calomel electrode, Radiometer #K4112

Micro-reaction chamber (0.5-4.0 ml functional capacity) with magnetic stirrer

Water bath

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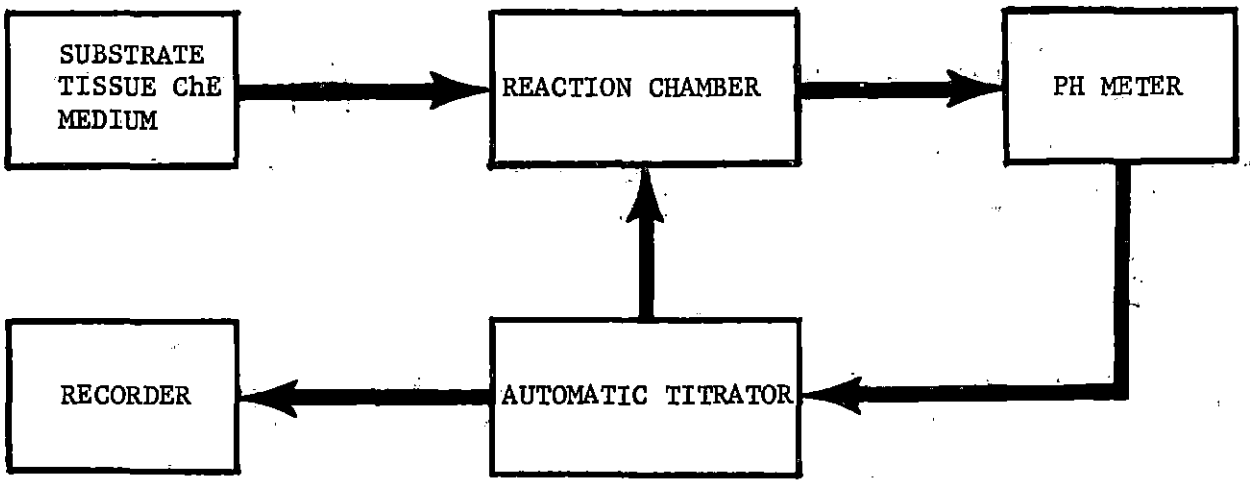
[1] Sigma Chemical Co. (ACh is stored under N_2 gas because of its hygroscopic nature.)

Block Diagram (pH-Stat Apparatus)



Procedure:

1. Remove brain samples as rapidly as possible and store in liquid N_2 until assayed. Begin ChE determination as soon as practical (usually within 1 or 2 days).
2. Set water bath at 30° C. Begin continuous stream of N_2 gas over contents of reaction chamber. Set end point of titration at pH of 7.6 and paper speed at 2 min/cm.
3. Calibrate the pH electrodes with two buffers (pH 7.00 and 4.01).
4. Weigh brain samples to the nearest mg and place in 10 volumes of 0.1M NaCl.
5. Homogenize by ultrasonic cell disruption and keep on ice until assayed.
6. Place 2.5 ml of the medium (at 30° C) and 0.1 ml of the tissue homogenate into the reaction chamber. (The temperature of the reaction chamber contents should be in equilibrium with the water bath within 1 minute.)

7. With constant stirring, bring the pH of the reaction chamber contents to 7.6 by manual addition of the titrant. Avoid overshooting the end point by adding the titrant very slowly as the pH approaches 7.6.
8. Set the apparatus on automatic titrate. (Titrant will then be added in small increments (0.1 to 0.3 ul) in response to a rise in pH above 7.6. Each addition of the titrant is recorded on the graph from which the amount of NaOH added per unit time is determined. Homogenized brain tissue typically has spontaneous acid liberation (SAL), and this activity will be indicated by the rate of titration prior to the addition of substrate (ACh·Cl).) Record the SAL rate until 4 or 5 minutes of linearity are obtained.
9. Add 0.1 ml of substrate (0.250M ACh·Cl) to the reaction chamber. (This results in a final substrate concentration of 9.3mM ACh·Cl in the reaction chamber. ChE in the tissue homogenate will hydrolyze the added ACh. For each mole of ACh hydrolyzed, 1 mole of titrant (NaOH) is needed to maintain a constant pH. Therefore, titration rate will be increased by ChE activity (see Notes).)
10. From the slope of the recorded line, determine the rate of NaOH titration before and after the addition of substrate. (The difference between the two titration rates is the rate due to ChE activity.)
11. Calculations. ChE activity is calculated by the following equation:

$$\frac{(\mu l\ NaOH/min)(\mu mole\ NaOH/\mu l)}{(ml\ tissue\ homog.)(g\ tissue/ml\ tissue\ homog.)} = \frac{\mu mole\ NaOH/min/g}{(tissue\ wet\ weight)}$$

Example of calculation:

ChE activity of control rat hippocampus =

$$\frac{(4.3\ \mu l/min - 0.9\ \mu l/min)(0.0200 \mu mole/\mu l\ NaOH)}{(0.100\ ml\ homog.)(1.00\ g\ tissue/11\ ml\ tissue\ homog.)} = 7.5\ \mu mole/min/g\ (NaOH)$$

Since 1 mole of NaOH is needed to neutralize the acid formed in the hydrolysis of 1 mole of ACh, the ChE activity is calculated to be:

ChE activity = 7.5 μmole/min/g (with ACh as substrate)

Notes:

1. The activity of brain ChE determined by this method is the total activity of all cholinesterases (i.e. acetylcholinesterase (AChE), butyrocholinesterase (BuChE), propionocholinesterase (PrChE), and nonspecific esterases) in the brain using ACh as a substrate. BuChE, PrChE, and nonspecific esterases, however, contribute only a small fraction of the total ACh-hydrolyzing activity found in rat brain (Koelle, 1954). Therefore, the ChE activity determined by this method is a valid indication of brain AChE activity.
2. The chemical reaction involved in the ChE-catalyzed hydrolysis of ACh is as follows:

$$ACh \xrightarrow{ChE} \text{acetic acid} + \text{choline}$$

One mole of NaOH is needed to neutralize the acid formed from each mole of ACh hydrolyzed in order to maintain a constant pH. The rate of NaOH titration due to ChE activity is the difference between the rate of NaOH titration <u>before</u> and <u>after</u> the addition of ACh. (ChE activity does not appear to significantly contribute

to SAL (spontaneous acid liberation), since the SAL rate is not affected by inhibition of ChE.)

3. Spontaneous (nonenzymatic) hydrolysis of substrate is assumed to be negligible. The ChE activity observed for brain tissue from an animal given high doses of DFP (3.0 mg/Kg, i.v. in rabbit), an irreversible ChE inhibitor, is calculated to be 0.0 μmole/min/g. This indicates that the measurement of spontaneous hydrolysis of ACh (unaided by ChE) is not detected using this procedure.
4. Maximum accuracy in the measurement of titration rate from a recorded slope is obtained when the recorded line (after substrate addition) is at a 45^{o} angle to the vertical. An approximate 45^{o} angle was obtained for the recording of titration rate of ChE activity in rat brain hippocampus (about 8 μmole/min/g) following the above pH-stat procedure. If measurements of ChE activity considerably greater than or less than 8 μmole/min/g are to be made, the paper speed and/or concentration of the titrant can be varied to bring the recorded titration rate line (after substrate addition) to the approximated 45^{o} angle.