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Irl Extein
Yale University

ACCUMULATION OF METABOLITES IN CSF: A MEASURE OF FUNCTIONAL CHANGES IN BRAIN DOPAMINE AND NOREPINEPHRINE METABOLISM IN RABBITS AND MAN

IRL LAWRENCE EXTEIN

1974

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Accumulation of metabolites in CSF:
a means of functional changes in brain
DA and NE metabolism

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Accumulation of metabolites in CSF: a measure of functional changes in brain dopamine and norepinephrine metabolism in rabbits and man

Irl Lawrence Extein

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the requirements for the degree of

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1974

Abstract:

This thesis represents the results of several related methods of measuring the accumulation of major metabolites of dopamine (DA) and norepinephrine (NE) in the brain and cerebrospinal fluid (CSF). These methods include measurement of the endogenous metabolites as well as measurement of the radioactivity of the metabolites following intravenous injection of labeled L-dopa, a precursor of the parent catecholamines. Peripheral decarboxylase inhibitors were administered prior to the injection of labeled L-dopa in order to minimize peripheral metabolism. Probenecid was administered in order to block the transport of these metabolites out of the brain and CSF. The metabolites studied were homovanillic acid (HVA), a major metabolite of brain DA, and 3-methoxy-4-hydroxyphenylglycol (MHPG) and its sulfate conjugate (MHPG-S), major metabolites of brain NE.

Chlorpromazine increased the probenecid-induced accumulation of endogenous and labeled HVA in rabbit brain and CSF. A decreased accumulation of endogenous and labeled HVA was measured in the lumbar CSF of humans with Parkinson's disease. A probenecid-induced accumulation of endogenous MHPG-S in rabbit brain and CSF was demonstrated. This accumulation of MHPG-S was enhanced by foot-shock stress and slightly decreased by chlorpromazine. Chlorpromazine also decreased the amount of labeled MHPG in rabbit brain. However, in rat brain the accumulation of MHPG-S was slightly increased by chlorpromazine.

These results indicate that the measured changes in CSF metabolites probably reflect functional changes in the metabolism

of DA and NE in the central nervous system. The role played by brain DA and NE in the mechanisms of action of antipsychotic drugs like chlorpromazine is discussed.

Acknowledgements:

I would like to thank Drs. Robert H. Roth, Malcolm B. Bowers, Jr., and Melvin H. Van Woert for their support in carrying out this research. Their scientific and technical knowledge was an invaluable resource and their willingness to share their time and offer their friendship made our relationship one I will cherish. Most of all I would like to thank them for allowing and encouraging my independence.

Many thanks also to Ms. Angelika Rozitis and Ms. Amanda Smith for their technical assistance.

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Contents:

Introduction:

Metabolic pathways for the synthesis and degradation of the catecholamines dopamine (DA) and norepinephrine (NE) have been known for about fifteen years (9) (see fig. 1). The physiology and pharmacology of these monoamines at their respective synapses have been extensively studied (37, 69, 70). They are thought to function as neurotransmitters. More recently discrete neuronal pathways which utilize either DA or NE as transmitters have been identified by the methods of fluorescence hisochemistry (130).

Both DA and NE are thought to play a role in the etiology and therapy of many forms of neurological and psychiatric disorders, including Parkinson's disease (40,68, 74), schizophrenia (75, 87, 110, 124, 125, 126, 137), and affective illnesses (30, 36, 57, 87, 117, 118, 119, 120, 121). It has been difficult to study direct electrophysiological and biochemical effects on the post-synaptic side of the neuronal synapses in the brain (3, 35, 26, 66, 114). However, functional changes, and particularly drug-induced changes, in the activity and metabolism of DA and NE neurons have been measured by a variety of methods. Changes in neuronal activity have been measured by single-cell recording of the firing of individual neurons (28, 29, 61). Changes in brain amine turnover have been measured by the rate of disappearance of DA and NE following synthesis inhibition (3, 5, 16, 18, 39, 79, 81, 88, 93), by isotopic labeling techniques (53, 95, 96), and by measurement of the levels of metabolites of the parent amines in the brain (1, 4, 6, 28, 31, 80, 81, 91, 94, 112), the cerebrospinal fluid (CSF) (20, 21, 22, 23,

24, 25, 32, 34, 36, 56, 57, 58, 59, 60, 64, 71, 72, 77, 78, 83, 97, 98, 100, 105, 106, 131, 134, 136), and urine (27, 48, 85, 86). Probenecid blocks the transport of certain weak acids out of the brain and CSF and has been used to study the accumulation of monoamine metabolites in the brain (89, 94, 135) and CSF (21, 22, 23, 58, 60, 68, 78, 97, 128) as an index of central turnover.

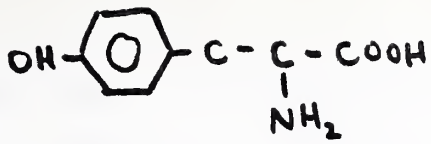
In humans, CSF is the accesible tissue which probably best reflects brain metabolism of monoamines. CSF levels of homovanillic acid (HVA---a major brain DA metabolite) and 5-hydroxyindoleacetic acid (5HIAA---a major metabolite of brain serotonin) have been extensively studied in a variety of pathological and drug-induced states in animals and man.

Many drugs affecting levels of metabolites of these amines in brain tissue have similar effects on CSF levels of these metabolites. It has been concluded that CSF amine metabolites originate in the brain and that changes in the probenecid-induced accumulation of these metabolites in the CSF are an index of changes in brain amine metabolism (23, 58, 77, 97). However, whether the rate of accumulation of HVA and 5HIAA in the CSF after probenecid loading is directly related to the rate of formation of these metabolites in the brain has yet to be established. 3-methoxy-4-hydroxyphenylglycol (MHPG) and its sulfate conjugate ((MHPG-S) are major metabolites of brain NE (88, 116). They have been much less extensively studied in CSF than have been HVA and 5HIAA (60, 136).

The work here presented represents an attempt to expand the methodology whereby brain DA and NE metabolism can be studied by measuring their metabolites in the brain and particularly in the CSF. The work will be presented in three parts.

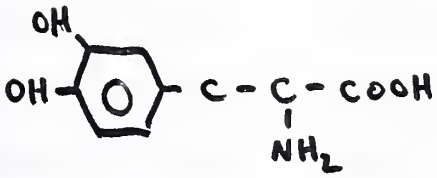
In part I the probenecid-induced accumulation of endogenous HVA in rabbit CSF was demonstrated. A new method was developed whereby the accumulation of ^{3}H-HVA could be measured in CSF following intravenous administration of ^{3}H-L-dopa to rabbits pretreated with a decarboxylase inhibitor and probenecid. Accumulation in the CSF of both endogenous and labeled HVA increased in response to changes in central DA metabolism induced by chlorpromazine. Part II demonstrates the application of the methods of Part I to the study of patients with Parkinson's disease, whose pathology is known to involve a defect in central DA neurons. Methods parallel to those used in Part I to study HVA, a major DA metabolite, have been applied in Part III to the study in rabbits of MHPG and its sulfate conjugate, major central NE metabolites. A probenecid-induced accumulation of endogenous MHPG-S was demonstrated for the first time in CSF. It was also shown that ^{3}H-MHPG appeared in brain following intravenous administration of labeled L-dopa. Accumulation of endogenous MHPG-S in rabbit brain and CSF was shown to increase in response to increases in central NE metabolism induced by foot-shock stress. The effect of chlorpromazine on the accumulation of endogenous MHPG-S and labeled MHPG in rabbit brain and CSF and in rat brain were studied as well.

Figure 1. Metabolic pathways for the synthesis and degradation of dopamine and norepinephrine in brain. TH=tyrosine hydroxylase. LAAAD=L-aromatic amino acid decarboxylase. DBH=dopamine beta-hydroxylase. MAO=monoamine oxidase. AR=aldehyde reductose (alcohol dehydrogenase). COMT=catechol-O-methyl transferase. HVA=homovanillic acid. MHPG=3-methoxy-4-hydroxyphenylglycol. AD=aldehyde dehydrogenase.



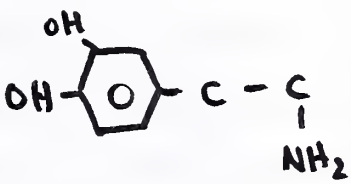
Tyrosine

TH



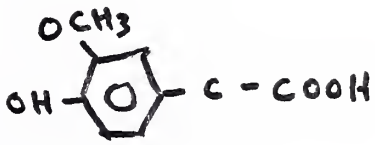
Dopa

LAAAD



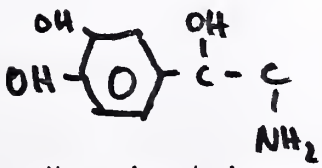
Dopamine

MAO → AD → COMT



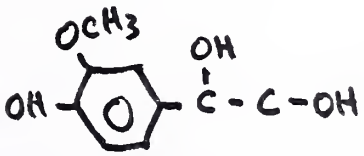
HVA

DBH

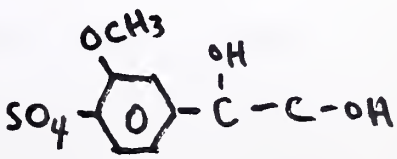


Norepinephrine

MAO → AR → COMT



MHPG



MHPG-sulfate

Part One: HVA in rabbit brain and CSF following i.v. ^{3}H-L-dopa--- the effect of chlorpromazine

Introduction:

Part I describes a method for pulse-labeling brain DA pools by systemic administration of labeled L-dopa. The subsequent accumulation in brain and CSF of labeled HVA, a major brain DA metabolite, may be an index of central dopaminergic neuronal activity.

Systemically administered L-dopa is thought to be converted to DA within the brain, which accounts for its efficacy in the treatment of Parkinson's disease (40). It has been demonstrated previously in animals (14) and humans (102) administered ^{14}C-L-dopa that ^{14}C-HVA is measurable several hours later in the CSF.

Utilizing intravenous administration of ^{3}H-L-DOPA to rabbits treated with a peripheral decarboxylase inhibitor and probenecid, we sought to study in animals the metabolic fate in blood, brain, and CSF of systemically administered L-DOPA. Accumulation of labeled and endogenous HVA was measured in rabbit brain and CSF. Probenecid-induced accumulation of endogenous HVA has been suggested to be a useful measure of brain DA metabolism in animals (21, 63) and man (23, 58, 77, 97).

Chlorpromazine, like other antipsychotic drugs, is known to increase brain DA metabolism (1, 3, 31, 95) and dopaminergic neuronal activity (28). Therefore, groups of rabbits were pretreated with this drug in order to determine if the measured accumulation of ^{3}H-HVA following ^{3}H-L-DOPA changed in response to known changes in brain DA metabolism.

Methods:

Male white rabbits weighing 2.0 - 2.5 kg were treated with RO4-4602 in a dose (50 mg/kg, i.p.) which inhibits L-aromatic amino acid decarboxylase extracerebrally (1, 12). ^{3}H-L-DOPA (100 μc of L-3, 4-dihydroxyphenylalanine ring 2,5,6 ^{3}H, specific activity 4 C/mmole, obtained from Amersham-Searle) was injected intravenously one-half hour later. The transport of HVA out of the CSF was blocked by a high dose of probenecid (150 mg/kg, i.p.) administered every two hours beginning ten minutes before the ^{3}H-L-DOPA injection. Control rabbits as well as drug pretreated rabbits were injected with probenecid. When the effects of drug-pretreatment were studied, the drug was administered immediately prior to the ^{3}H-L-DOPA injection. Chlorpromazine (10 mg/kg) was injected intravenously.

At a given time after ^{3}H-L-DOPA injection, each rabbit was anesthetized with ether. Blood was obtained by cardiac puncture and 0.5-1.0 ml of CSF was obtained by cisternal puncture. The brain was then removed and the subcortex dissected out. The subcortex consisted of midbrain, diencephalon, and basal ganglia and weighed approximately 2.0 g.

Serum metabolites of ^{3}H-L-DOPA were separated on aluminum oxide and Dowex 50 columns (52).

Brain tissue was deproteinized by homogenization with 8 ml of 0.4 N perchloric acid and then neutralized with potassium hydroxide to precipitate the perchlorate. Brain supernatants and CSF samples were then freeze-dried and taken up in 80% ethyl alcohol. The volume was reduced by evaporation in a stream of

nitrogen gas before being spotted on cellulose thin-layer chromatographic plates for analysis by a two-dimensional method (49). Standards of DOPA, DA, and HVA were included in the spot, so that after development of the plates, each substance could be visualized, removed, and its radioactivity determined. Radioactivity was measured by scintillation counting in 10 ml of a solution made by dissolving 5.5 g PPO and 300 mg POPOP in 2 l toluene and adding 1 liter of Triton X-100. Efficiency was determined by individual internal ^{3}H-toluene standards.

Because of the limited amount of CSF which could be obtained from each rabbit, levels of endogenous HVA in brain and CSF were determined fluorometrically (51) in a second, separate experiment with rabbits not injected with ^{3}H-L-DOPA. These rabbits received the exact schedule of probenecid injections and chlorpromazine pretreatment as did the rabbits in the first experiment. Apparent specific activities of CSF HVA were calculated by dividing the radioactivity measured in the first experiemtn by the endogenous levels measured in the second experiment, at the same time points.

Results:

Serum analysis showed that the aluminum oxide eluate fraction (DOPA, DA, DOPAC) declined rapidly after one-half hour, whereas the Dowex 50 eluate fraction containing O-methyl-DOPA increased greatly up to one hour.

The time course for the appearance of ^{3}H-L-DOPA and its metabolites in brain and CSF is shown in Figures 2 and 3. Brain DA radioactivity was maximal one-half hour after injection of ^{3}H-L-DOPA and declined rapidly in amounts consistent with the subsequent statistically significant accumulation of ^{3}H-HVA in brain

and CSF.

The time course of the probenecid-induced accumulation of endogenous HVA in CSF is shown in Figure 3. For the first several hours the increase of endogenous HVA and ^{3}H-HVA were approximately parallel. Hence the calculated apparent specific activities were approximately constant over this time period. Endogenous HVA did not accumulate in rabbit brain following probenecid (Table 1).

Figure 4 shows that chlorpromazine pretreatment caused a significant two to three-fold increase in the amount of ^{3}H-HVA accumulated in brain and CSF by four hours, which was paralleled by a similar increase in the accumulation of endogenous HVA in CSF. The calculated apparent specific activity of HVA in the CSF four hours after ^{3}H-L-Dopa injection was not affected by chlorpromazine pre-treatment (Figure 4).

Discussion:

Results of the analysis of L-DOPA metabolites in serum confirm the efficacy of the decarboxylase inhibitor. ^{3}H-L-DOPA was present in highest concentratrations in the blood for one-half hour, and was then rapidly O-methylated, but only slowly decarboxylated or deaminated.

The time course of the appearance of ^{3}H-HVA in CSF is in basic agreement with earlier studies in humans using ^{14}C-L-DOPA in the absence of a decarboxylase inhibitor or probenecid (28). The plateau maintained in CSF between two and four hours is consistent with the blockade of HVA egress from CSF by probenecid. ^{3}H-HVA reached a sharper and an earlier maximum in brain than it

did in CSF, and then declined. This decline is consistent with the finding (13,135) that probenecid does not effectively inhibit HVA egress from rabbit brain. The failure of HVA to increase in rabbit brain following probenecid has been reported previously (135). Concentrations of endogenous HVA are high in rabbit brain, suggesting that HVA transport out of brain is inefficient in this species and not dependent on a probenecid-sensitive active transport. Why HVA fails to accumulate in rabbit brain but accumulates in rabbit CSF following probenecid is not well understood (see part III).

The appearance of ^{3}H-L-DOPA and ^{3}H-DA in brain and their sharp fall from one-half to two hours after injection of ^{3}H-L-DOPA are certainly consistent with the assumption that ^{3}H-DA is formed in the brain from exogenously administered ^{3}H-L-DOPA, becomes part of a functional endogenous DA pool, and then is metabolized. Over the same time period ^{3}H-HVA increased significantly in brain and CSF, in parallel with the increase in endogenous CSF HVA. Hence the formation of ^{3}H-HVA from ^{3}H-DA seems to reflect the same processes whereby endogenous HVA is formed from endogenous DA.

However, it is not certain that either all the endogenous HVA in CSF or all the labeled HVA appearing in CSF after administration of exogenous labeled L-DOPA represents DA released from dopaminergic neurons and subsequently metabolized to HVA. Endogenous HVA might be made from DA formed in brain capillaries (17). However, it has been demonstrated in rats (Roth, R.H., unpublished data), cats and monkeys (27; 98), and humans with Parkinson's disease (26, 97) that lesions affecting the cellularity of the

substantia nigra cause parallel decreases in striatal and CSF HVA. Stimulation of the substantia nigra of the cat causes large increments in the amount of HVA appearing in lateral ventricular CSF (30,).

Some portion of ^{3}H-HVA in rabbit CSF following even small doses of ^{3}H-L-DOPA i.v. might represent capillary metabolism, despite peripheral decarboxylase inhibition (14). In the dose used here, RO4-4602 does inhibit the decarboxylase localized in brain capillary walls without interfering with the same enzyme in the cerebral parenchyma (12), and should at least minimize the contribution of capillaries. It should be pointed out that should ^{3}H-HVA be formed outside the CNS, it has been shown to penetrate the brain quite poorly (11, 102). Another possible artifactual source of HVA following exogenous L-DOPA is via O-methyl-dopa. But only a very small percentage of blood O-methyl-dopa is converted into brain amines (15).

Even if non-neuronal metabolism is minimized, other neurons besides dopaminergic neurons can decarboxylate exogenous L-DOPA to DA (82) and lead to HVA formation. However, labeled L-DOPA administered i.v. (101) or intraventricularly (54, 55) has resulted in an uneven distribution of labeled amines in the brain, roughly parallel to the distribution of endogenous catecholamines. Preliminary reports in the monkey demonstrate that the dopaminergic nigro-striatal pathway must be intact in order for administration of exogenous L-DOPA to raise CSF HVA significantly (98),

The fact that chlorpromazine induced the same increase in brain and CSF ^{3}H-HVA measured by our method as it induces in endogenous HVA is, in our opinion, the best evidence for the

assumption that the ^{3}H-HVA detected here in CSF after exogenous ^{3}H-L-DOPA administration represents *in vivo* brain neuronal DA metabolism. The increase in endogenous HVA in the striatum (1) and CSF (25, 64, 100) following phenothiazine-like drugs is thought to reflect a compensatory increase in brain DA turnover in response to blockade of post-synaptic DA receptors (31). This increased DA turnover is probably at least in part a reflection of the reported increased rate of firing of dopaminergic neurons following chlorpromazine and similar drugs (28). The chlorpromazine-induced acceleration of DA synthesis from ^{14}C-tyrosine (95) has been shown to be abolished by lesions of the dopaminergic nigro-striatal neuronal pathways (96).

Table 1.

The effect of probenecid on HVA levels in rabbit brain.

Levels of homovanillic acid (HVA) in rabbit subcortex before and two hours after treatment with probenecid, 150 mg/kg i.p. Each value is the mean in ng/g of brain tissue ± S.E.M. for the number of rabbits indicated in parentheses.

time after probenecid treatment in hours	homovanillic acid in subcortex
0	2.345 ± 133 (8)
2	2.015 ± 168 (7)

Figure 2. Levels of labeled L-DOPA, dopamine (DA), and homovanillic acid (HVA) in the subcortex at various times after intravenous administration af ^{3}H-L-DOPA to rabbits treated with RO4-4602 and probenecid. Each point is the mean disintegrations per minute (DPM) per gram of brain tissue $\pm$ S.E.M. for the number of rabbits indicated in parentheses. ^{3}H-L-DOPA (o——o), ^{3}H-DA (x——x), ^{3}H-HVA (●——●). (*) indicates a significant increase above levels of ^{3}H-HVA in subcortex at one-half hour, $p < 0.05$ by two-tailed t-test.

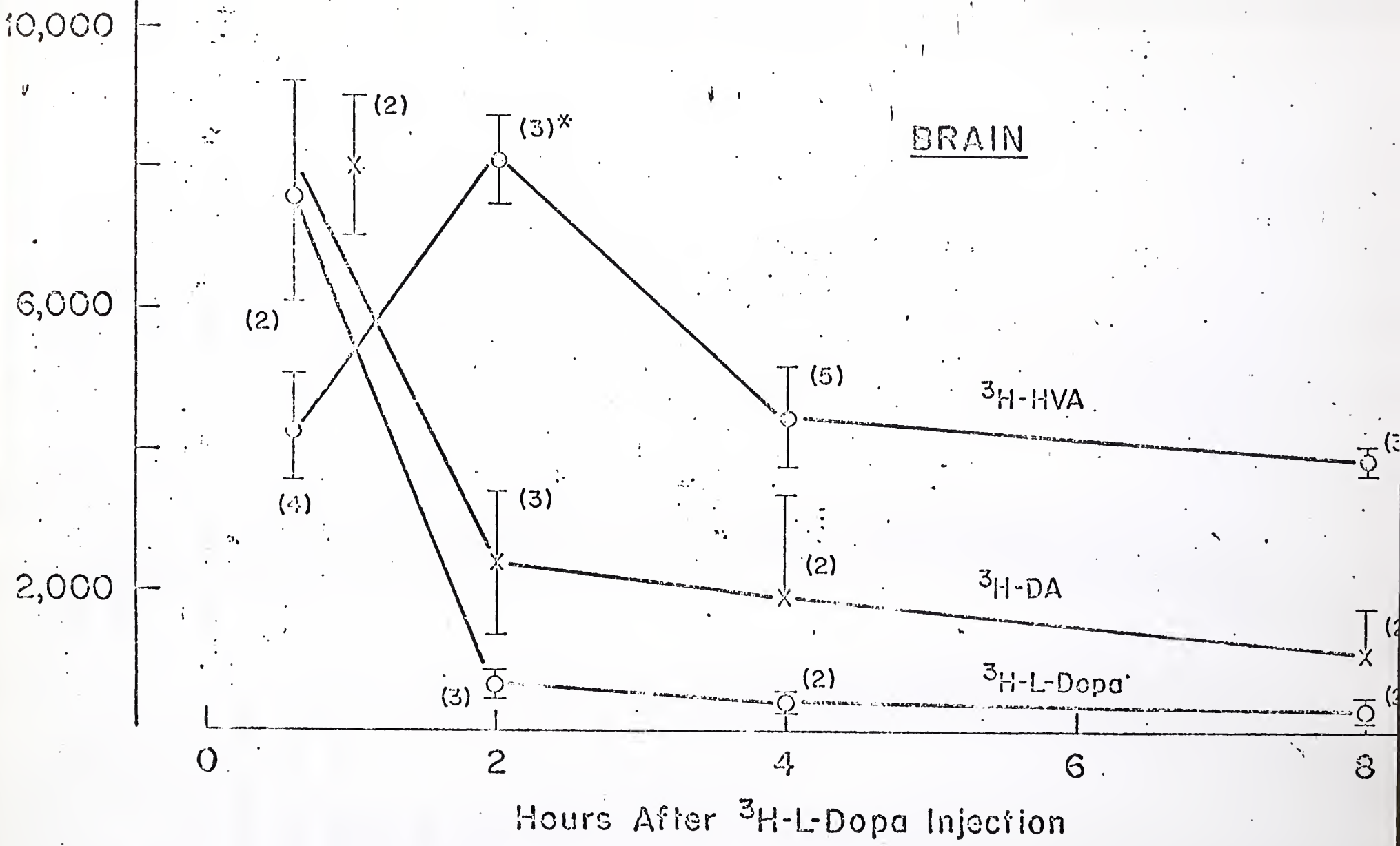

10,000
6,000
2,000
BRAIN
(2)
(3)*
(2)
(5)
(4)
(3)
(2)
(3)
(2)
^{3}H-HVA
^{3}H-DA
^{3}H-L-Dopa
0
2
4
6
8
Hours After ^{3}H-L-Dopa Injection

Figure 3. Levels of labeled and endogenous homovanillic acid (HVA) in the cisternal cerebrospinal fluid (CSF). ^{3}H-HVA was measured at various times after intravenous administration of ^{3}H-L-DOPA to rabbits treated with RO4-4602 and probenecid. Endogenous HVA was measured in a separate experiment with different rabbits treated with the same probenecid schedule. Each point is the mean disintegrations per minute (DPM) of ^{3}H-HVA, or ng of endogenous HVA, per ml of CSF $\pm$ S.E.M. for the number of rabbits indicated in parenthesis. ^{3}H-HVA (o——o), endogenous HVA (△——△). Significant increases above levels of ^{3}H-HVA in CSF at one-half hour (*) or above levels of endogenous HVA in the CSF of untreated controls (**) are indicated. $p < 0.01$ by two-tailed t-test.

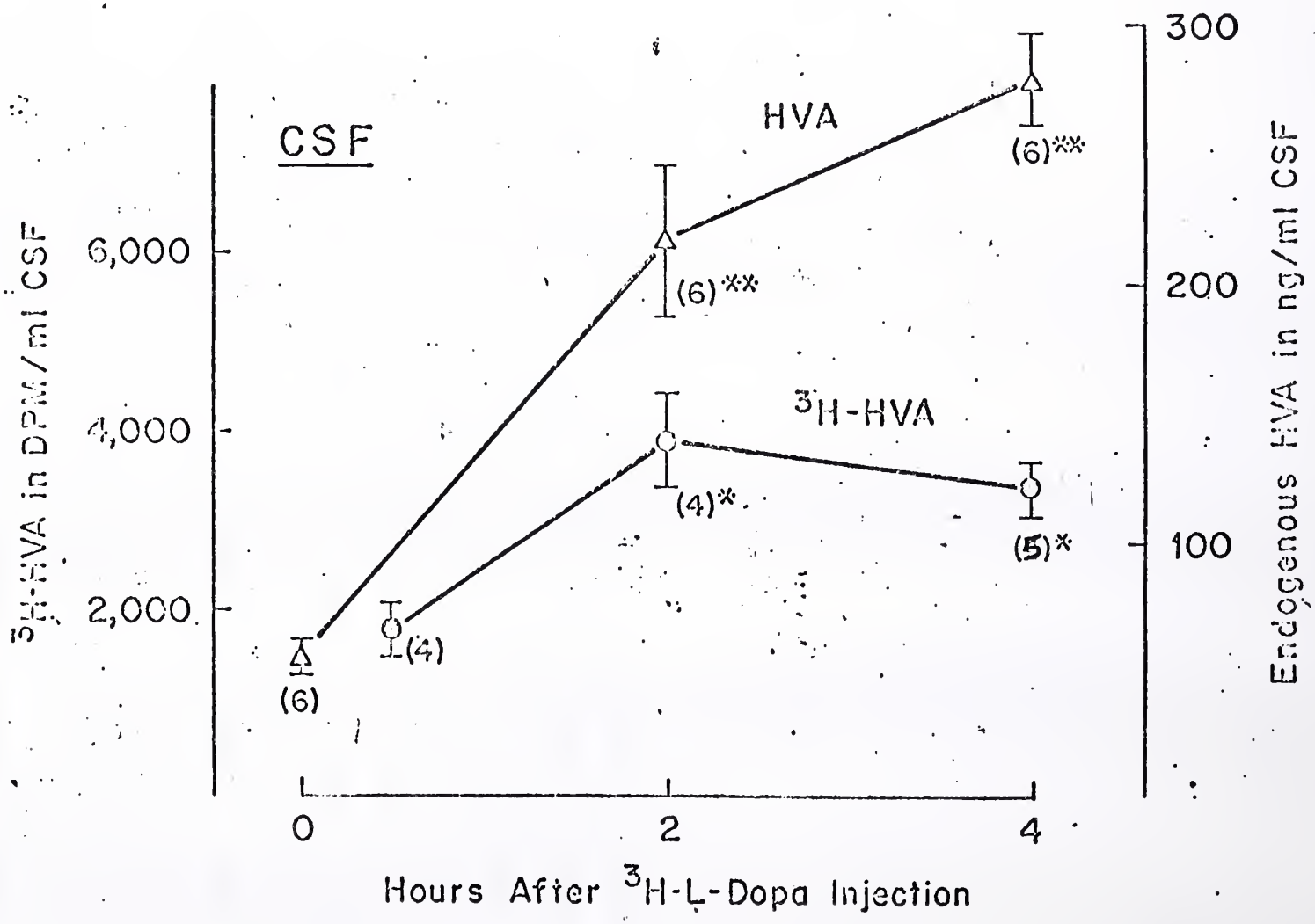
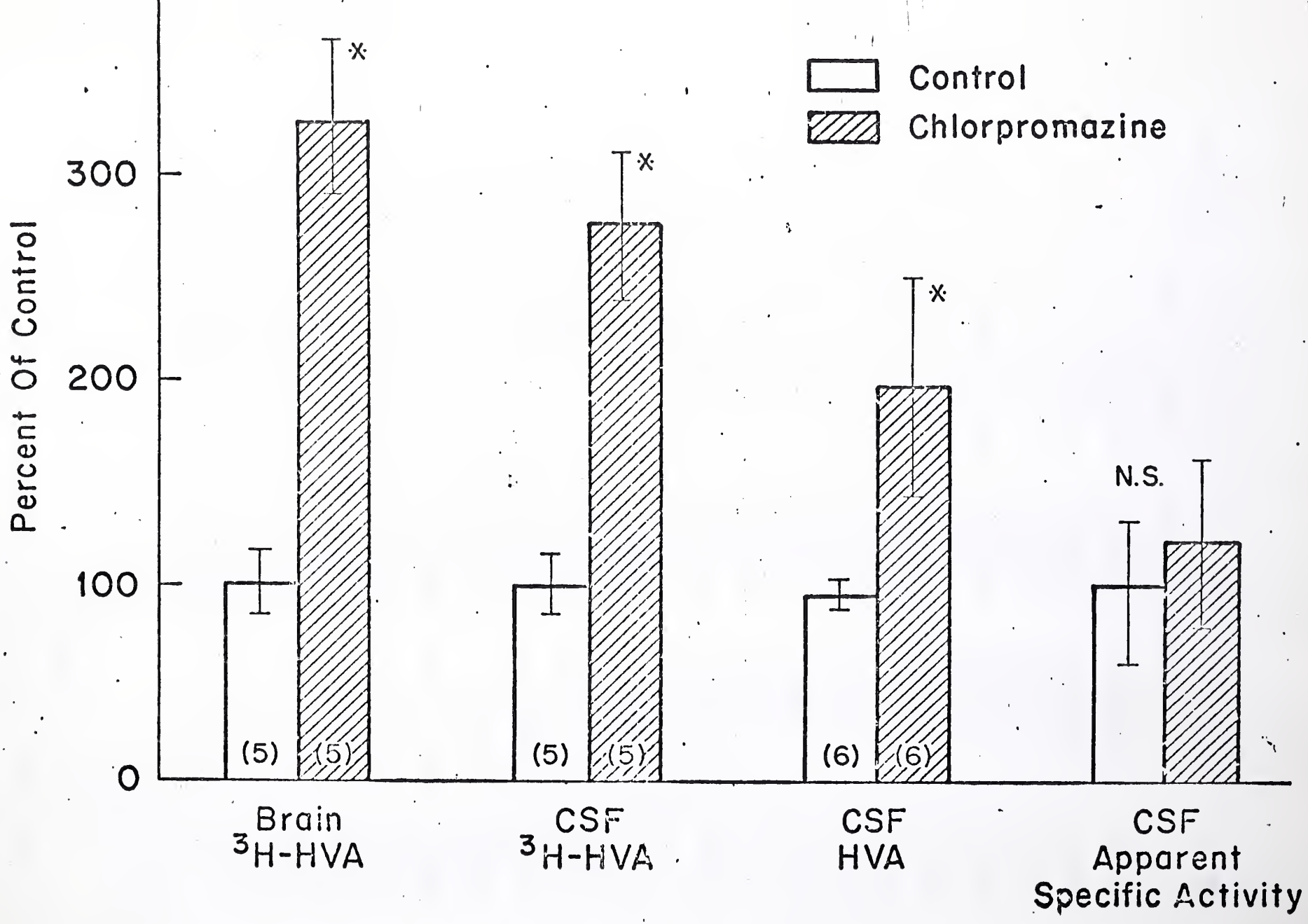

CSF
HVA
^{3}H-HVA
(6)**
(6)**
(4)*
(5)*
(6)
(4)
6,000
4,000
2,000
300
200
100
0
2
4
^{3}H-HVA in DPM/ml CSF
Endogenous HVA in ng/ml CSF
Hours After ^{3}H-L-Dopa Injection

Figure 4. Levels of labeled and endogenous homovanillic acid (HVA) in the subcortex and cisternal cerebrospinal fluid (CSF). ^{3}H-HVA was measured four hours after intravenous administration of ^{3}H-L-DOPA to rabbits treated with R04-4602 and probenecid. Endogenous HVA was measured in a separate experiment with different rabbits treated with the same probenecid schedule for four hours. Apparent specific activity of HVA in CSF at four hours was calculated by dividing the levels of ^{3}H-HVA in disintegrations per minute/ml at four hours by the level of endogenous HVA in ng/ml at the same time point. Values in rabbits pretreated with chlorpromazine (10 mg/kg i.v.) are expressed as the mean percentage of controls $\pm$ S.E.M. for the number of rabbits indicated in parentheses. Control and chlorpromazine pretreated rabbits received the same schedule of R04-4602 and probenecid injections. Significant drug-induced increases are noted by (*) for $p < 0.01$ by two-tailed t-test.


Control
Chlorpromazine
Percent Of Control
300
200
100
0
*
*
*
N.S.
(5)
(5)
(5)
(5)
(6)
(6)
Brain
^{3}H-HVA
CSF
^{3}H-HVA
CSF
HVA
CSF
Apparent
Specific Activity

Part Two: HVA in the CSF of Parkinsonian patients following i.v. ^{14}C-L-dopa

Introduction:

Part II describes an application in humans of the methods of part I for pulse-labeling brain DA pools by intravenous administration of labeled L-dopa. The subsequent accumulation of HVA in CSF may be a clinically useful index of the central metabolism of L-dopa and of central dopaminergic neuronal activity. In animals, labeled tyrosine has been used to study synthesis and metabolism of DA and NE (95, 96), However, because of the participation of tyrosine in numerous metabolic pathways, it would be neither economical nor safe to administer labeled tyrosine to humans for the purpose of labeling brain catecholamines.

L-dopa has been found to be of therapeutic benefit in the treatment of Parkinson's disease (40). This effect is thought to be related to L-dopa's conversion to DA within the brain resulting in facilitation of dopaminergic transmission (68, 74). Many investigators have interpreted increased levels of HVA in CSF of Parkinsonian patients treated with exogenous L-dopa as an index of the hypothesized activation of dopaminergic neuronal pathways (32, 56, 123, 134). Use of a peripheral decarboxylase inhibitor potentiates L-dopa's therapeutic effects and probably minimizes the extra-neuronal contribution to the HVA appearing in the CSF after administration of exogenous L-dopa to patients (33).

There is some precedent for use of labeled exogenous L-dopa to study the metabolism of L-dopa and DA in humans. ^{14}C-L-dopa

has been given i.v. to humans followed by detection of ^{14}C-HVA in cisternal CSF several hours later (102). The work reported in this thesis makes several additions to the earlier methodology. CSF was obtained from the lumbar CSF---more distant from the brain but much more accesible. A peripheral decarboxylase inhibitor was used in an effort to decrease the extra-neuronal metabolism of the labeled L-dopa and increase the liklihood that the labeled HVA in CSF represented central metabolism of DA. Probenecid was used in order to try to measure the rate of formation of HVA instead of a static level. Parkinsonian patients were compared to controls in order to determine in humans if a known abnormality in brain dopaminergic systems would be reflected by the rate of formation of ^{14}C-HVA from ^{14}C-L-dopa, as was demonstrated in chlorpromazine treated rabbits in part I. It was hypothesized that if the HVA measured in the CSF reflected brain DA metabolism, then both endogenous and labeled HVA should be lower in patients with Parkinson's disease than in controls.

The pathology of Parkinson's disease is thought to involve a functional deficit in dopaminergic transmission in the striatum (68). Brains of Parkinsonian patients show a characteristic degeneration in the substantia nigra (68). This degeneration includes some of the DA neurons which project from the substantia nigra to the striatum. Post-mortum samples of Parkinsonian brain tissue have low levels of DA and HVA (68). The levels of CSF HVA (34, 56, 71, 97, 131), as well as the accumulation of HVA in the CSF following probenecid (24, 34, 83, 97), are also abnormally low in Parkinsonian patients. Extrapyramidal symptoms similar to those seen in parkinsonism can be produced by

drugs such as chlorpromazine which block DA receptors (75).

Measurement of probenecid-induced accumulation of labeled and endogenous HVA in CSF after a small dose of labeled L-DOPA might have certain advantages over measurement of endogenous HVA alone after probenecid. Individual variability in transport of HVA and the difficulty in humans in establishing sufficient concentrations of probenecid to completely block acid transport pose difficult problems to clinical research (23, 77). Specific activities of isolated HVA would be independent of the rate of egress of HVA from the CSF and may allow one to circumvent these problems. Certainly changes of labeled and endogenous HVA in the same direction would strengthen any conclusions based on the probenecid test in humans, and might better reflect the changes in brain DA activity that have been suggested in the etiology and therapy of certain neurological and psychiatric disorders (75, 110, 124).

Methods:

This study was performed according to a protocol approved by the Yale Clinical Investigations Committee and the Yale Radioisotope Committee. ^{14}C-L-dopa has been administered to humans by other investigators in doses of 100 μCi orally (99) and 5 μCi/kg i.v. (102). The ^{14}C was almost entirely excreted in urine within one week of i.v. administration of ^{14}C-L-dopa to humans (personal communication from Professor R. Tissot, Univ. of Geneva). MK-486 or alpha-methyldopahydrazine (13, 18, 104) (Carbidopa; Merck, Sharp, and Dohme) was used under an IND from the U.S. Food and Drug Administration. Informed consent was obtained from all participants.

Patients were studied on in-patient wards of the Yale Medical Center. Four were previously diagnosed Parkinsonians. Of the four controls, three were neurology patients with the diagnoses of dystonia muscularum deformans, multiple sclerosis, and cerebral palsy. One of the controls was a paid volunteer without active disease. The mean age of the Parkinsonians was 67, and the mean age of the controls was 48. The Parkinsonian group consisted of two males and two females, while the controls consisted of four males. All patients had been off all medication for several weeks prior to the admission.

The patients were given a peripheral decarboxylase inhibitor, MK-486, 50 mg po every six hours, beginning at 7 AM on the day prior to the injection of ^{14}C-L-dopa. This dose of MK-486 is higher than the amount commonly used to enhance the therapeutic effects of L-dopa in Parkinsonians (32, 33). The decarboxylase inhibition following oral administration of MK-486 peaks in about 3 or 4 hours (personal communication---Dr. M. Jaffe, Merck, Sharpe, and Dohme). It has been shown that a single dose of 100 mg po or daily doses of 100 mg po for one week are equally efficacious in inhibiting the peripheral decarboxylation of exogenously administered L-dopa (18).

Probenecid (Benemid; Merck, Sharpe, and Dohme) was given in a dose of one gram po at 5 PM of the day prior to injection of ^{14}C-L-dopa. This dose was repeated every four hours until CSF was obtained 24 hours after the first dose. Total dosage of probenecid was 6 g po in 24 hours, or about 100 mg/kg. This probenecid schedule(25) has been shown to cause accumulation of HVA in the lumbar CSF of humans. Patients were kept flat in bed from the

first dose of probenecid until the lumbar puncture (106).

50 μCi of ^{14}C-L-dopa [L-3 (3,4-dihydroxyphenyl)alanine-3-^{14}C, specific activity 21 mCi/mmol, supplied by Amersham-Searle] was injected intravenously at 9 AM, two hours after the last dose of MK-486. The labeled L-dopa was injected in 10 ml of sterile saline.

Seven and one-half hours later a lumbar puncture was performed and 10 ml of CSF was obtained and immediately put on ice. The timing of the lumbar puncture was considered optimal in order to detect the peak accumulation of ^{14}C-HVA in CSF. Following i.v. labeled L-dopa, labeled HVA has been reported to peak about 2-4 hours later in the cisternal CSF of rabbits (14) and humans (102). Endogenous HVA has been reported to begin accumulating in human lumbar CSF about 4 hours after oral administration of probencid (128). This last figure presumably represents the time for ventricular and cisternal HVA to begin to appear in lumbar CSF. The two lag-times were added in order to arrive at the estimate of 7.5 hours as the optimal time.

The CSF was centrifuged to remove any red blood cells and stored at -20° C. Endogenous HVA and 5HIAA were assayed by fluorescence methods (25). CSF probenecid levels were assayed by a spectrophotometric technique (77). Total radioactivity was determined by counting 0.1 ml of raw CSF. Radioactivity of ^{14}C-HVA was determined by a modification of the methods described in Part I. The procedure was revised in order to be able to do thin-layer chromatographic analysis on up to 5 ml of human CSF. The method consisted of deproteinization of 4 ml of CSF by acidification with perchloric acid, precipitation of the perchlorate

with potassium hydroxide, and extraction of non-polar interfering substances into pet ether before freeze-drying of the aqueous phase. Dopa, DA, and metabolites were extracted, and separated by thin-layer chromatography (49), and radioactivity of ^{14}C-HVA was counted by methods described in Part I. Samples were counted for 40 minutes, until the raw background counts reached about 1000. Efficiency was determined by individual internal ^{14}C-toluene standards. Background was determined by running cold CSF through the assay. Recovery of ^{14}C-HVA was determined by analysis of cold human CSF to which a known amount of ^{14}C-HVA standard was added. Recovery was 61%.

Results:

The results showed this procedure to be technically feasible in humans. Raw counts in the HVA spots ranged from 150 to 350 percent of background count. Although no time course was done, the $7\frac{1}{2}$ hour time after ^{14}C-L-dopa injection seems to be a usable time point for measuring ^{14}C-HVA accumulation in the lumbar CSF. Results are summarized in figure 5.

Endogenous HVA levels and HVA radioactivity were lower in the parkinsonian group than in controls. Specific activity of HVA, obtained by dividing the $7\frac{1}{2}$ hour accumulation of labeled HVA by the 24 hour accumulation of endogenous HVA, in each individual, was also lower in the parkinsonian group. Thus, the results are in the predicted direction.

The difference between ^{14}C-HVA in the two groups was more marked if the HVA radioactivity was expressed as a percentage of the total radioactivity in the CSF for each individual. This

reflects variance among individuals in the total radioactivity in CSF, perhaps related to incomplete decarboxylase inhibition in the periphery causing variance in the amount of ^{14}C-L-dopa reaching the brain. ^{14}C-HVA expressed as percentage of total CSF radioactivity may be the most accurate measure of what proportion of the ^{14}C-L-dopa reaching the brain is converted to ^{14}C-HVA.

Discussion:

Interpretation of the meaning of changes in the accumulation in CSF of endogenous HVA and labeled HVA formed from intravenously administered labeled L-dopa were discussed in part I. They probably reflect at least in part changes in central DA metabolism and dopaminergic neuronal activity. Use of the peripheral decarboxylase inhibitor decreases the proportion of ^{14}C-HVA formed by extra-cerebral and capillary metabolism of the ^{14}C-L-dopa (13, 18, 33).

There was much variation in the probenecid levels obtained. Levels of endogenous HVA were linearly related to the CSF probenecid levels. A schedule of probenecid administration similar to the one used here has been reported by others (25) to result in higher CSF probenecid levels and slightly higher CSF HVA levels. However, even at these higher probenecid levels, HVA levels were directly related to probenecid levels, indicating that HVA egress was not completely blocked. The dosage of probenecid that can be used is limited by production of nausea and vomiting. In the results shown in figure 5, CSF probenecid levels in the parkinsonian group were not significantly different from control. There was a tendency for higher probenecid values in the parkinsonian group,

which might be expected to bias the results in favor of higher HVA levels in this group. Thus, the variance in probenecid levels can not account for the lower levels of endogenous and labeled HVA in the CSF of parkinsonian patients.

Although the parkinsonians were older as a group, age has little effect on HVA levels.

Post-mortum studies of the brains of parkinsonians has shown DA and HVA in the basal ganglia and substantia nigra to be reduced by between 50 and 90 percent of controls (68). Measurement of HVA in the CSF of parkinsonians has consistently documented a reduction, but not to the extent of the reduction in the brain (71, 97, 131, 34, 56). Reports of decreased accumulation of HVA in the CSF of parkinsonian patients during probenecid treatment have been interpreted as reflecting a decreased turnover of central DA (24, 34, 83, 97). There is no agreement on whether the probenecid test makes differences between CSF HVA levels in parkinsonians and controls easier to detect (34, 97). Reports of the extent of the decreased HVA accumulation in the CSF of parkinsonians treated with probenecid vary from a 70% decrease (97) to small and statistically insignificant decreases (24). There is also disagreement as to whether the extent of the decrease in CSF HVA is correlated with severity of illness or clinical response to L-dopa therapy (24, 34, 83).

The differences reported here in endogenous levels are only 20%. Variance in probenecid levels and small sample may have kept this from being significant. The 50% decrease in ^{14}C-HVA is consistent with other reports of decreased HVA in the CSF of parkinsonians. Thus, the formation of labeled HVA from labeled

DA seems to reflect the same process whereby endogenous HVA is formed from endogenous DA. Presumably, the labeled HVA was formed from labeled brain DA physiologically released from dopaminergic neurons (see part I). The decrease in labeled HVA in the CSF of parkinsonians could represent a decrease in the formation or utilization of DA at any step along the path from L-dopa to HVA. Like the decrease in endogenous HVA, it may reflect an abnormality in DA neurons, or may reflect the decreased number of normal neurons caused by degeneration in the nigra-striatal pathway. Thus, the decreased formation of ^{14}C-HVA from ^{14}C-L-dopa seems to reflect the anatomical and functional abnormalities in DA neurons which are known to exist in Parkinson's disease.

The decreased specific activity reported here in parkinsonians may be important. If it is real, then it implies that formation of labeled HVA from labeled L-dopa is more sensitive to the pathological changes in Parkinson's disease than is the formation of endogenous HVA. Perhaps a larger proportion of endogenous HVA in the CSF comes from peripheral sources and masks the deficiency in central DA turnover.

In conclusion, it has been shown here that it is clinically feasible to administer tracer amounts of labeled L-dopa intravenously to humans premedicated with a peripheral decarboxylase inhibitor and probenecid and to detect easily measurable amounts of labeled HVA subsequently in the lumbar CSF. The fact that the amount of ^{14}C-HVA accumulating in the CSF of patients with Parkinson's disease is decreased supports the assumption that changes in ^{14}C-HVA accumulation in CSF reflect functional changes in brain DA metabolism.

Figure 5. Endogenous and labeled homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5-HIAA), and probenecid in the lumbar cerebrospinal fluid (CSF) $7\frac{1}{2}$ hours after intravenous injection of 50 uCi of ^{14}C-L-dopa to humans premedicated with the peripheral decarboxylase inhibitor alpha-methyl-dopa hydrazine and probenecid. Values are expressed as the mean $\pm$ S.E.M. for the number of patients indicated in parentheses.

	HVA ng/ml	5HIAA ng/ml	Probenecid ug/ml	^{14}C-HVA dpm/ml	^{14}C-HVA as % of total ^{14}C in CSF	HVA Specific Activity dpm/ng
Controls	112±32(4)	57 (2)	7.4±1.9(4)	26.7±8.0(3)	2.8±0.8(3)	.36±.15(3)
Parkinsonians	92±31(4)	69 (3)	10.0±3.2(4)	18.0±3.5(4)	1.3±0.4(4)	.24±.07(4)

Part Three: MHPG in rabbit brain and CSF

In part III a probenecid-induced accumulation of MHPG-S in rabbit brain and CSF is reported. A method is described in rabbits for labeling brain NE by i.v. administration of labeled L-dopa and measuring the subsequent appearance of labeled MHPG in brain. The accumulation in brain and CSF of MHPG, a major brain NE metabolite, may be an index of central noradrenergic neuronal activity. These methods in part III parallel the methods used in part I for measuring HVA as an index of dopaminergic activity.

Adequate methods for measuring the metabolism of biogenic amines in the human brain will undoubtedly contribute to the knowledge of the function of these amines. Some recent progress has been made regarding the metabolism of DA and serotonin (5-hydroxytryptamine or 5HT) in the human brain. Probenecid-induced accumulation of the metabolites HVA and 5HIAA in the CSF of human patients has already been used as a hypothesized measure of abnormalities in the central metabolism of DA and serotonin respectively. However, a similar approach for the metabolism of NE in the human or animal brain has not yet been described (23, 58, 60, 77, 97).

IIIA: A probenecid-induced accumulation of MHPG-sulfate in the brain and CSF

Introduction:

The sulfate ester of 3-methoxy-4-hydroxyphenylglycol, MHPG-S, is a major central nervous system (CNS) metabolite of NE (84, 113, 116). It has been demonstrated in rat(74) that MHPG-S levels in cerebral cortex are dependent on the functional integrity of ascending NE-pathways. Total MHPG, determined after hydrolysis of MHPG-S, has been measured in the CSF of animals (109, 115) and humans (59, 136) by gas chromatography. In the rat labeled MHPG-S injected intraventricularly is transported out of the CSF by a probenecid-sensitive mechanism (83). Results using a fluorometric assay (90) to determine endogenous levels of MHPG-S show that this metabolite accumulates in rat brain following probenecid (91). However, at the time we did this study, there were no published data describing a probenecid-induced increase in endogenous MHPG-S in the CSF of any animal. Preliminary studies (58, 59) in human CSF had failed to detect any such increase. We report here a marked probenecid -induced increase of MHPG-S in rabbit CSF and brain.

Methods:

White rabbits weighing about 2.0 kg were maintained in a ventilated 30.5°C box. They maintained a constant rectal temperature. Following ether anesthesia brain and CSF samples were obtained without any drug treatment in some animals. Other rabbits were treated with probenecid---150 mg/kg i.p., repeated

every two hours. Either two or four hours after the first probenecid injection, these rabbits were killed by ether inhalation and 0.5-1.5 ml of CSF was quickly withdrawn by cisternal puncture, placed on ice, and then frozen. The brain was then removed, and the cerebral cortex and a portion of the subcortex were dissected out and frozen. The portion of subcortex was a piece of the midbrain weighing about 400 mg, chosen because of its proximity to the ventricular CSF.

MHPG-S and NE were determined in the same sample of brain tissue. Tissue was homogenized in 0.4 N perchloric acid and the MHPG-S was isolated on DEAE sephadex anion exchanger and determined by a fluorometric method (9, 11, 80, 90). We collected the effluent and the washing fluid (5 ml of 0.08 N HCL), mixed it with 1 ml of 10% ethylenediamine tetracetate, and determined NE fluorometrically (111). The amount of MHPG-S in brain was expressed as the ratio of MHPG-S to NE in order to minimize individual variation in the brain tissue samples. In order to measure CSF MHPG-S, 0.5 ml of CSF was mixed with 2 ml of water and 0.5 ml of 1% formic acid. The MHPG-S was isolated and determined fluorometrically (80, 90). In the HVA investigations HVA was assayed (51) in subcortex and in a 0.5 ml sample of CSF.

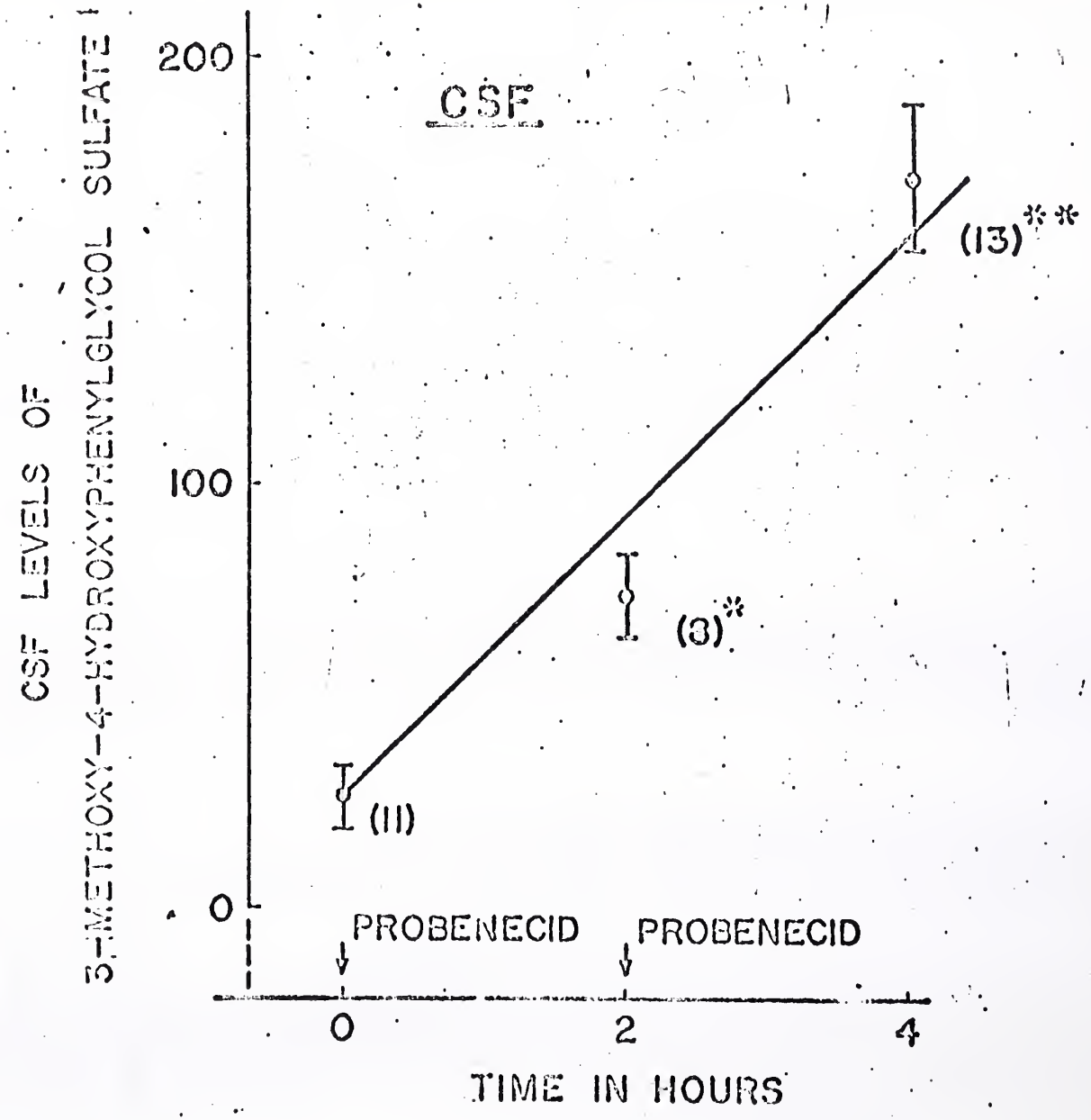
Results and Discussion:

Control levels of MHPG-S were 27 $\pm$ 6 ng/ml of CSF (n = 11), 87 $\pm$ 17 ng/g of cerebral cortex (n = 4), and 120 $\pm$ 14 ng/g of midbrain (n = 4). Control levels of NE were 207 $\pm$ 27 ng/g of cerebral cortex (n = 4), and 307 $\pm$ 13 ng/g of midbrain (n = 4).

There was a significant nearly linear increase in MHPG-S in the CSF of rabbits treated with probenecid for four hours (fig. 6). After four hours the levels were increased about sixfold above controls. These results are consistent with the findings of Meek and Neff (89) who showed that in rat the egress of intraventricularly administered radioactive MHPG-S was blocked by probenecid.

In both cerebral cortex and midbrain (fig. 7) there was a significant increase in the MHPG-S to NE ratio after two hours of probenecid treatment. However, there was no significant change from two to four hours (fig. 7), at a time when MHPG-S continued to increase in the CSF. It is likely that the blockade in the egress of MHPG-S from brain was not complete in the rabbits, despite the high probenecid dosage. In rats, doses up to 400 mg/kg are required to block MHPG-S egress from brain (91). The differential effect of probenecid on MHPG-S in rabbit brain and CSF could involve inability to attain sufficient tissue concentrations of probenecid, differences in the sensitivity of CSF and brain transport mechanisms, or that egress of a part of the MHPG-S from brain occurs by passive diffusion or bulk flow once certain high concentrations of the metabolite have built up in brain tissue. In any case, there appears to be a differential effect of probenecid on the accumulation of MHPG-S in brain tissue and CSF, and therefore, one must be careful in drawing conclusions about MHPG-S production in brain based on the probenecid-induced accumulation of this metabolite in the CSF. It should be noted in this regard that in the rabbit, probenecid causes an increase in HVA in CSF, but not in brain (see Table 1). 5HIAA also has been reported to increase faster in rabbit CSF than in brain following probenecid (22).

Figure 6. Levels of 3-methoxy-4-hydroxyphenylglycol-sulfate (MHPG-S) in rabbit cisternal CSF before and two and four hours after treatment with probenecid (150 mg/kg, i.p., repeated every two hours). Each value is the mean in ng/ml $\pm$ S.E.M. for the number of rabbits indicated in parentheses. * denotes a level significantly greater than the level before probenecid treatment, $p \leq 0.001$, by the two-tailed t-test. ** denotes a level significantly greater than the levels before and two hours after probenecid treatment, $p \leq 0.001$, by the two-tailed t-test.


CSF
CSF LEVELS OF
3-METHOXY-4-HYDROXYPHENYLGLYCOL SULFATE
200
100
0
(11)
(8)*
(13)**
PROBENECID
PROBENECID
0
2
4
TIME IN HOURS

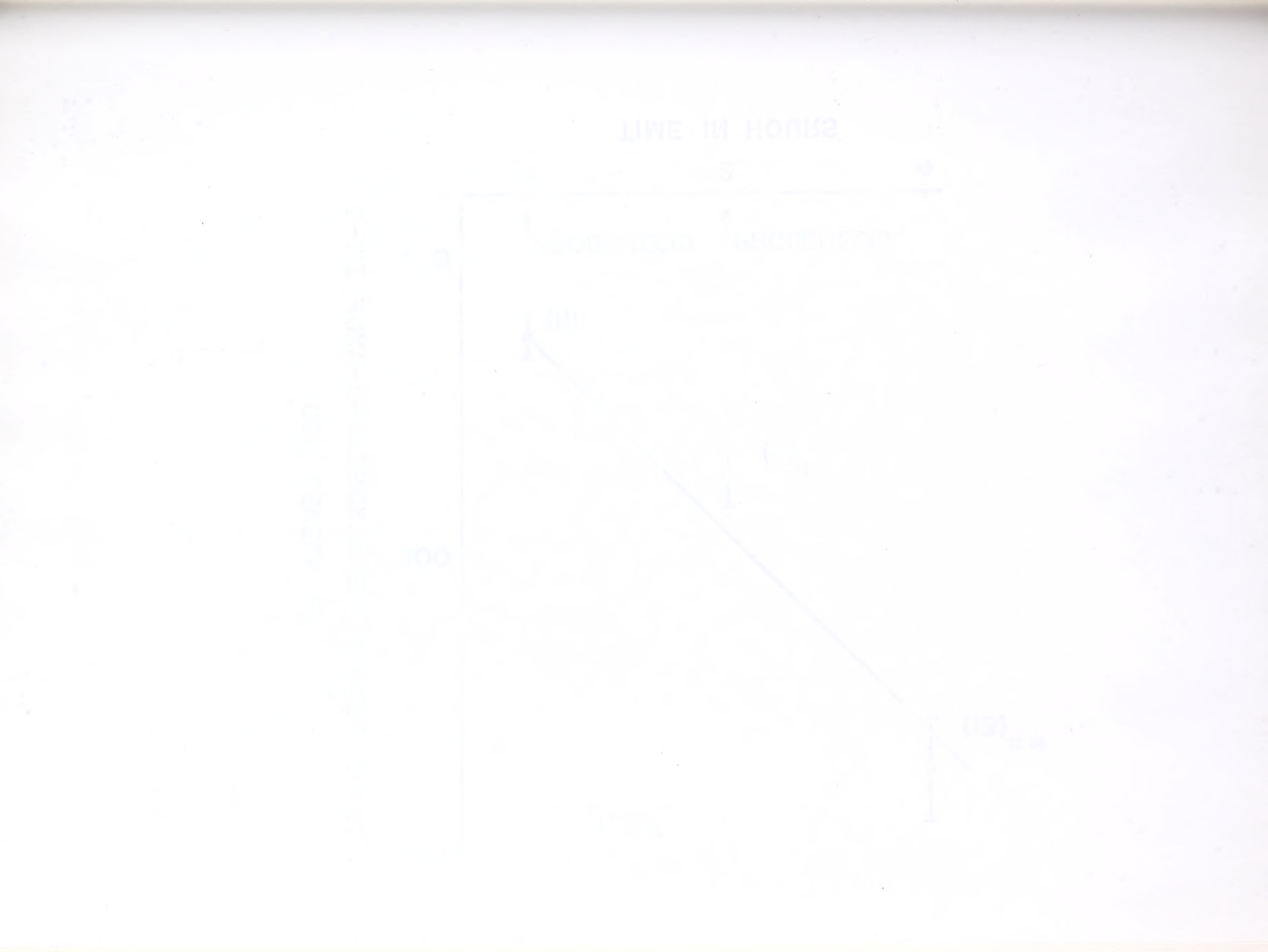
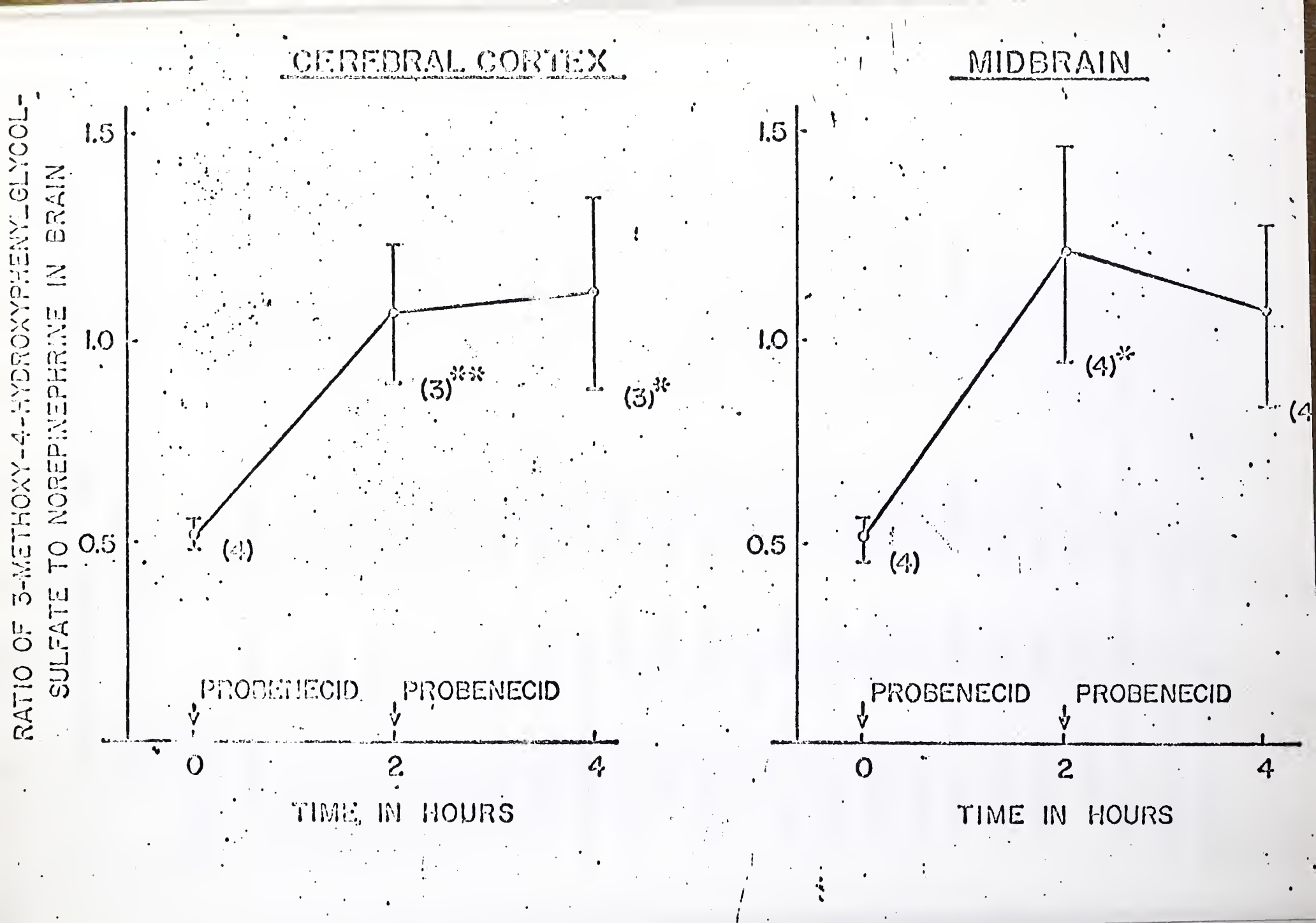


Figure 7. Ratios of 3-methoxy-4-hydroxyphenylglycol-sulfate (MHPH-S to norepinephrine (NE) in the cerebral cortex and midbrain of rabbits before and two and four hours after treatment with probenecid (150 mg/kg i.p., repeated every two hours). Each value is the mean $\pm$ S.E.M. for the number of rabbits indicated in parentheses. Ratios significantly greater than the ratio before probenecid treated are noted by (*) for $p \leq 0.05$ and by (**) for $p \leq 0.01$, by the two-tailed t-test.


CEREBRAL CORTEX
MIDBRAIN
RATIO OF 3-METHOXY-4-HYDROXYPHENYLGLYCOL-SULFATE TO NOREPINEPHRINE IN BRAIN
1.5
1.0
0.5
(4)
(3)**
(3)*
(4)*
PROBENECID
PROBENECID
0
2
4
TIME IN HOURS

IIIB: The effects of stress and chlorpromazine on the probenecid-induced accumulation of MHPG-sulfate in brain and CSF

Introduction:

In order to determine if the probenecid-induced accumulation of MHPG-S in brain and CSF reflects central noradrenergic neuronal activity, the effect of "stress" was measured. A variety of stress procedures have been shown in animals to enhance brain NE turnover (39, 50, 81, 88). Accumulation of HVA in brain and CSF has been shown to change in response to changes in brain DA metabolism and turnover, as demonstrated in parts I and II. Similarly, it might be expected that the accumulation of MHPG-S in brain and CSF would change in response to a change in brain NE metabolism such as that caused by foot-shock stress.

Recently it has been reported (81) that electric foot-shock increases NE turnover and MHPG-S levels in rat brain. These effects were mediated via increased impulse flow in the nucleus locus coeruleus, a group of NE-containing neurons in the brainstem which project to more rostral structures (81). If probenecid-induced accumulation of MHPG-S in CSF also reflects central NE metabolism it would be expected that electric foot-shock "stress" would increase this accumulation. Results reported here demonstrate that foot-shock increases the probenecid-induced accumulation of MHPG-S in rabbit brain and CSF.

Antipsychotic drugs such as chlorpromazine are thought to act as blockers of post-synaptic DA and NE receptors (see Part I and Discussion). Chlorpromazine has been shown by a variety of methods to increase the turnover of DA and NE in brain. This

increase is thought to be a compensatory response by pre-synaptic neurons to be post-synaptic receptor blockade. However, the evidence for increased DA turnover is better established than is the evidence for increased NE turnover following chlorpromazine (see Discussion). If chlorpromazine increases NE turnover in the brain in the same way that it increases DA turnover, one might expect to measure an increase in NE metabolites in brain and CSF similar to the increase in the DA metabolite HVA measured in Part I. It has recently been reported that a spectrum of antipsychotic drugs, including chlorpromazine, caused a significant increase in MHPG-S in rat brain (72). Phenoxybenzamine, an alpha adrenergic blocker in the periphery but not an antipsychotic drug, has been shown to increase the probenecid-induced accumulation of MHPG-S in the rat brain (91).

Methods:

In the shock-stress experiments white rabbits weighing approximately 2.5 kg were injected with probenecid, 150 mg/kg i.p. Fifteen minutes later the rabbits' feet were shaved and they were placed in a plexiglass box with metal grid floor. The floor was connected via a scramble to an electrical shock generator (Grason-Stadler Model # E1100EA) programmed to generate shocks of 5 mamps intensity and 1 second duration every 10 seconds (81). The rabbits were shocked for 1.75 hours, removed from the box, and 0.5 hours later, they were anesthetized by ether inhalation. Control rabbits were injected with probenecid and kept in their home cages for 2.5 hours until being anesthetized. A sample of 0.5-1.5 ml of CSF was obtained by cisternal puncture, and

the brain was removed and dissected into cerebral cortex and subcortex. The cerebral cortex included the hippocampus and weighed approximately 4.5 g. The subcortex consisted of basal ganglia, diencephalon, and midbrain and weighed about 2 g. Brain and CSF MHPG-S were assayed by a fluorescence method (80, 90) described previously in part IIIA.

In the chlorpromazine experiments white rats weighing about 300 g and white rabbits weighing about 2.5 kg were maintained in a ventilated 30.5°C box, so that the animals' rectal temperatures remained normal throughout all experiments. Sixteen rats were injected with probenecid, 200 mg/kg i.p. Five minutes later, half of these rats were injected with chlorpromazine, 10 mg/kg i.p. Two hours later all sixteen rats were decapitated, their brains removed, and the caudate nuclei dissected out. The caudate nuclei of pairs of chlorpromazine-treated rats and those of pairs of control rats were pooled and assayed for HVA. The rest of the brain was dissected into cerebral cortex and subcortex as described previously for assay of MHPG-S. Ten rabbits were injected with probenecid, 150 mg/kg every two hours. Five rabbits were pretreated with chlorpromazine 10 mg/kg i.v. two hours before the first probenecid injection. Four hours after the first probenecid injection, all ten rabbits were killed by ether inhalation, and CSF and brain tissue were obtained and MHPG-S assayed (80, 90) as described previously.

Results:

Results of the shock and stress experiments are recorded in figure 8. The shock procedure caused a statistically significant

increase in the accumulation of MPHG-S in cerebral cortex and CSF compared to controls.

Results of the chlorpromazine experiments are summarized in figures 9 and 10. Chlorpromazine induced a small but statistically significant increase in the accumulation of MHPG-S in the rat cerebral cortex and a non-significant increase in the MHPG-S accumulation in the rat subcortex. In the rabbit subcortex and cerebral cortex chlorpromazine induced a non-significant decrease in the accumulation of MHPG-S. There was a statistically significant decrease in the accumulation of MHPG-S in rabbit CSF following chlorpromazine pretreatment. Similar chlorpromazine pretreatment was shown in Part I (see figure 4) to double the accumulation of HVA in rabbit CSF in four hours.

Discussion:

Stimulation of the locus coeruleus in the rat has been shown to cause an increase in NE turnover following synthesis inhibition (79) as well as to increase levels of MHPG-S in the brain (80). These effects are abolished by lesions in the locus. Thus, in the rat brain MHPG-S seems to reflect noradrenergic neuronal activity and NE metabolism. A schedule of electric foot-shocks similar to that used here in rabbits has been shown in rats to increase brain NE turnover following synthesis inhibition (81). This increased turnover correlated with an increased level of MHPG-S in the brain. Both effects were abolished by lesions of the NE neurons in the locus coeruleus. Thus, the increased MHPG-S in the brain of stressed rats seems to reflect activation of NE neurons and increased brain NE metabolism.

The results reported here in rabbits indicate that foot-shock stress increases brain and CSF MHPG-S. The approximately 25% increases are less than might be expected from the severe stress procedure used here, and less than reported from similar shock schedules in the rat (81). Perhaps if unconjugated MHPG-S (115) had been measured, or if higher doses of probenecid were used (91), the increase would be greater. If as in the rat this increased MHPG-S reflects activation of brain NE neurons, then the data suggest that functional changes in noradrenergic neuronal activity and NE metabolism in rabbit brain are reflected in changes in the probenecid-induced accumulation of MHPG-S in brain and CSF. However, studies of the effect of stimulation and lesion of brain NE cells are needed to document the source and significance of NE metabolites in rabbit CSF.

Chlorpromazine in the dose of 10 mg/kg i.p. has been reported in rats to increase whole brain MHPG-S by about 20 percent after two hours (72). The results reported here for probenecid-treated rats injected i.v. with the same chlorpromazine dose of 10 mg/kg show a similar 20 percent increase in brain MHPG-S after two hours. It is difficult to say why chlorpromazine slightly increases MHPG-S in the rat and slightly decreases levels of this metabolite in the rabbit. The sulfate conjugate is known to be the major NE metabolite in rat brain (115, 116), but the ratio of conjugated to unconjugated MHPG in rabbit brain has not been studied. Perhaps increases in free MHPG in rabbit brain went undetected in these experiments.

It does seem clear that in the dose of 10 mg/kg chlorpromazine does not have a big effect on MHPG-S levels in either the

rat or the rabbit. However, HVA levels are greatly increased. Much higher doses of chlorpromazine up to 50 mg/kg i.p. have been reported to raise MHPG-S only 40 percent in the rat brain (72). This small effect is difficult to explain in terms of the theory that chlorpromazine and other antipsychotic drugs increase the activity of DA and/or NE neurons as a feed-back response to blockade of post-synaptic receptors. Chlorpromazine is felt to block both NE and DA receptors (3), and hence one would expect it to increase levels of DA and NE metabolites. Chlorpromazine and other antipsychotic drugs cause marked increases in HVA levels in rats and rabbits (1). Some antipsychotic drugs other than chlorpromazine have been reported to double MHPG-S levels in rat brain (72). It should again be noted that the chlorpromazine-induced increase in NE turnover measured by other methods is less pronounced and less consistently documented than the increase in DA turnover (see Discussion).

There are several possible explanations for the relative lack of effect of chlorpromazine on MHPG-S levels. It may be that the egress of MHPG-S must be completely blocked in order to detect an increase in rate of formation. It has been reported (91) that doses of up to 400 mg/kg are needed to block MHPG-S egress in the rat. The dose used here in rabbits and rats was 150 and 200 mg/kg respectively. However, other antipsychotic drugs more than double MHPG-S in rats who received no probenecid (72). It may be that properties peculiar to chlorpromazine make its effects on NE metabolism difficult to detect by measurement of MHPG-S. It should be mentioned that chlorpromazine has been shown to inhibit the liver alcohol dehydrogenase (73). If the related enzyme in

brain, the aldehyde reductase (see figure 1) is similarly inhibited then NE metabolism might be shunted away from MHPG formation and toward VMA. the main NE metabolite in the periphery (84). This might explain why chlorpromazine had little effect on the accumulation of MHPG in the rat and rabbit. Chlorpromazine could also interfere with sulfate conjugation.

In summary, stress procedures which are known to increase brain NE metabolism increase the probenecid-induced accumulation of MHPG-S in rabbit brain and CSF. Thus, the accumulation of MHPG-S in the CSF might be a useful index of central noradrenergic function. Chlorpromazine slightly increased the accumulation of MHPG-S in rat brain and slightly decreasd the accumulation in rabbit brain and CSF. The relatively small effect of chlorpromazine on MHPG-S levels is discussed in terms of current hypotheses about the DA and NE receptor blocking action of this drug.

This investigation suggests that it might be possible to measure increases in MHPG-S in human CSF following probenecid. At approximately the same time that the probenecid-induced increase in MHPG-S in rabbit CSF was reported from our laboratory (47), another group of investigators reported small increases in MHPG and MHPG-S in the lumbar CSF of patients treated with probenecid 100 mg/kg p.o. for eighteen hours (60). This accumulation is much less marked than that of HVA and 5-HIAA, perhaps because larger doses of probenecid are needed to block MHPG-S transport (91). It should also be kept in mind that about half the MHPG in human CSF is not the conjugated from but the free form (60) whose transport does not seem to be probenecid sensitive (89). If it is possible in humans to block MHPG-S transport to a

significant degree, then the accumulation of this metabolite might be a useful measure of the abnormalities in central noradrenergic function thought to be involved in some neurological and psychiatric disorders (30, 117).

Figure 8. Levels of 3-methoxy-4-hydroxyphenylglycol-sulfate (MHPG-S) in the brain and cerebrospinal fluid (CSF) of rabbits 2.5 hours after injection with probenecid, 150 mg/kg i.p. Some of the rabbits were given electric foot-shocks, 5 mA intensity, 1 second duration, every 10 seconds for 1.75 hours following the probenecid injection. Each bar-graph represents the mean in ng/g brain or ml CSF for the number of rabbits indicated in parentheses. Significant shock-induced increases are noted by (**) for $p < 0.05$ and by (*) for $p < 0.02$, by two-tailed t-test.

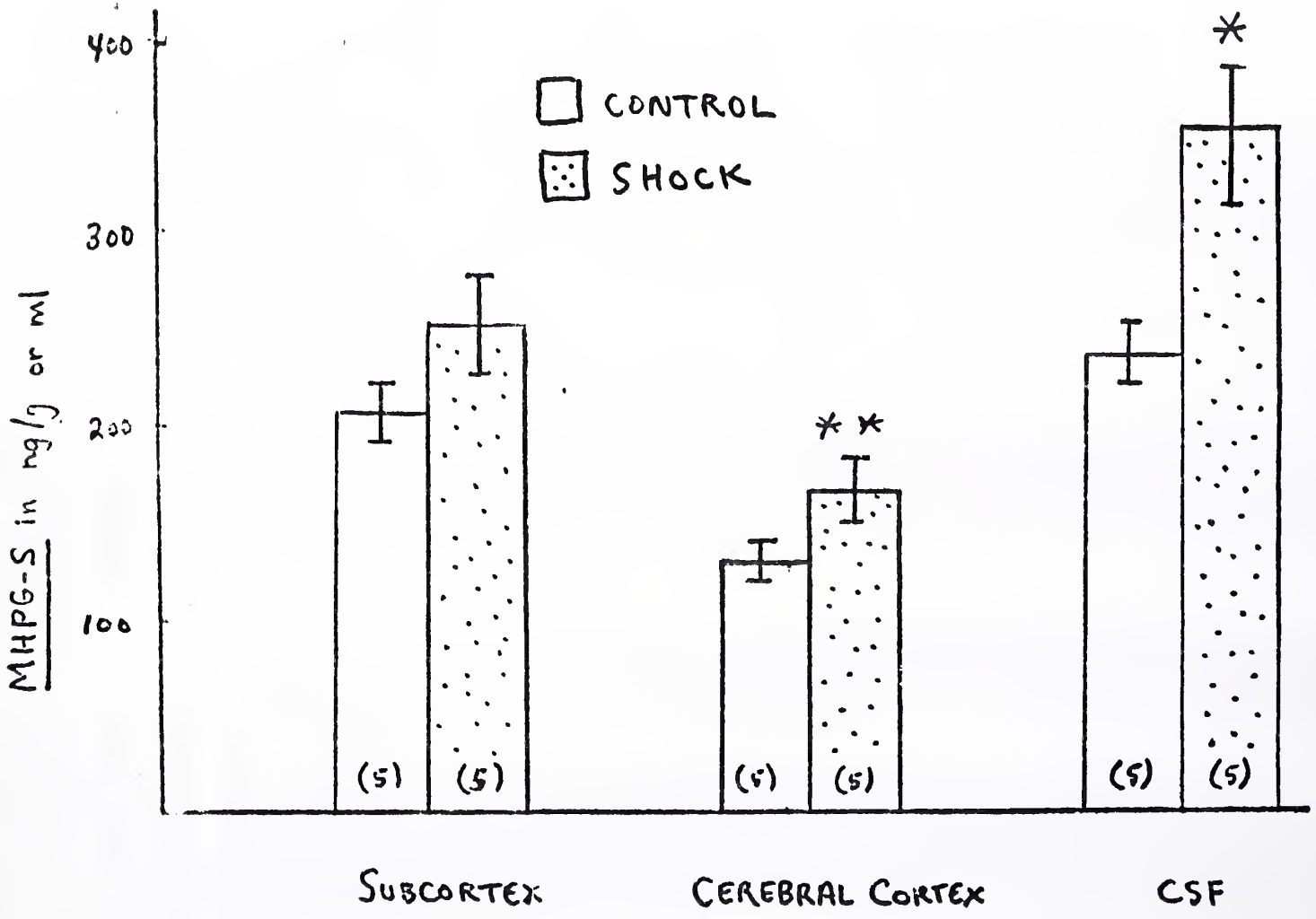

400
300
200
100
MHPG-S in ng/g or ml
CONTROL
SHOCK
* *
*
(5)
(5)
(5)
(5)
(5)
(5)
SUBCORTEX
CEREBRAL CORTEX
CSF

Figure 9. Levels of 3-methoxy-4-hydroxyphenylglycol-sulfate (MHPG-S) and homovanillic acid (HVA) in the brain of rats 2 hours after injection of probenecid, 200 mg/kg i.p. Some of the rats were treated with chlorpromazine, 10 mg/kg i.p. five minutes after the probenecid injection. Each bar graph represents the mean ng/g brain $\pm$ S.E.M. for the number of rats indicated in parentheses. Significant chlorpromazine-induced changes are noted by (*) for $p < 0.05$ by two-tailed t-test.

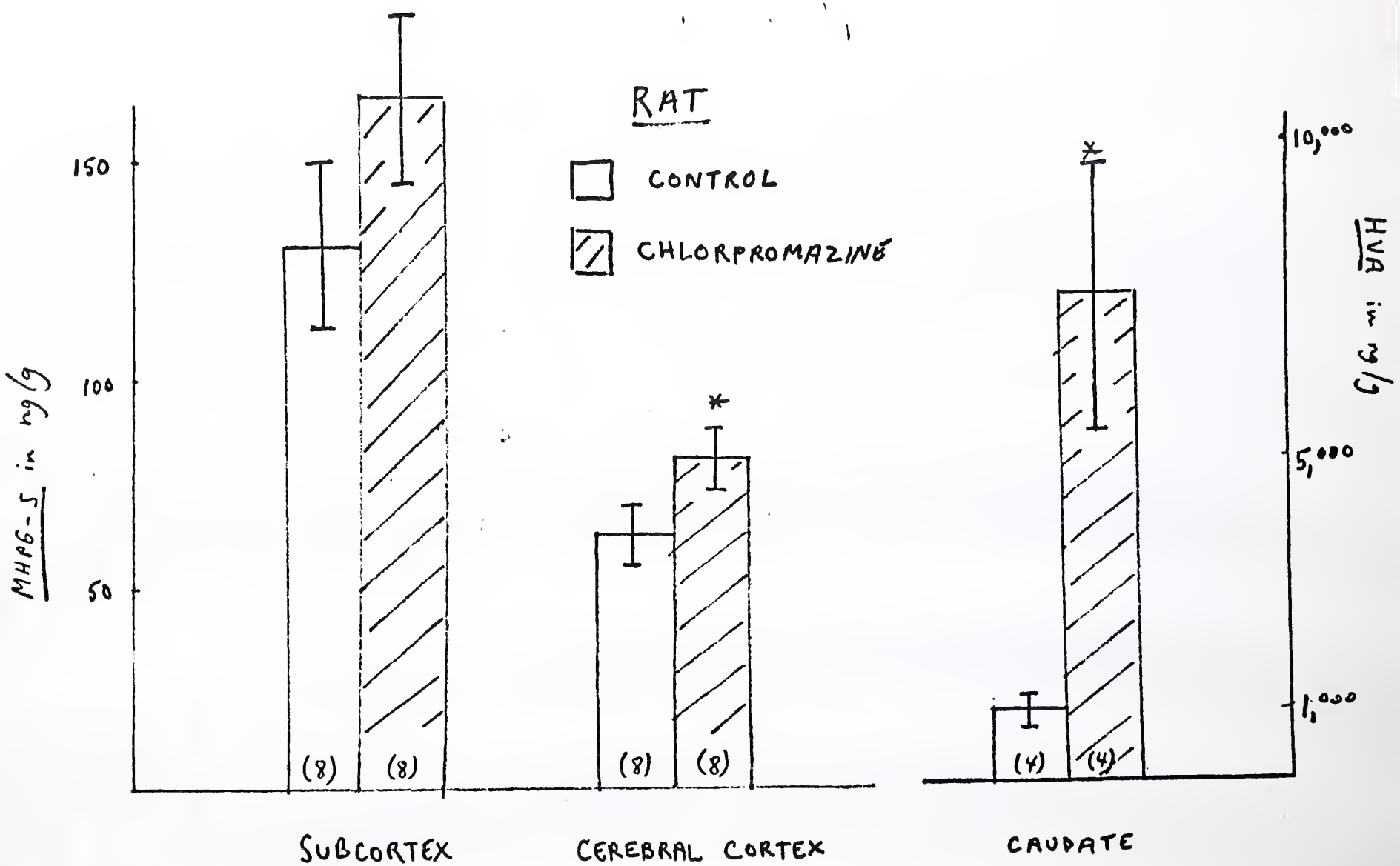
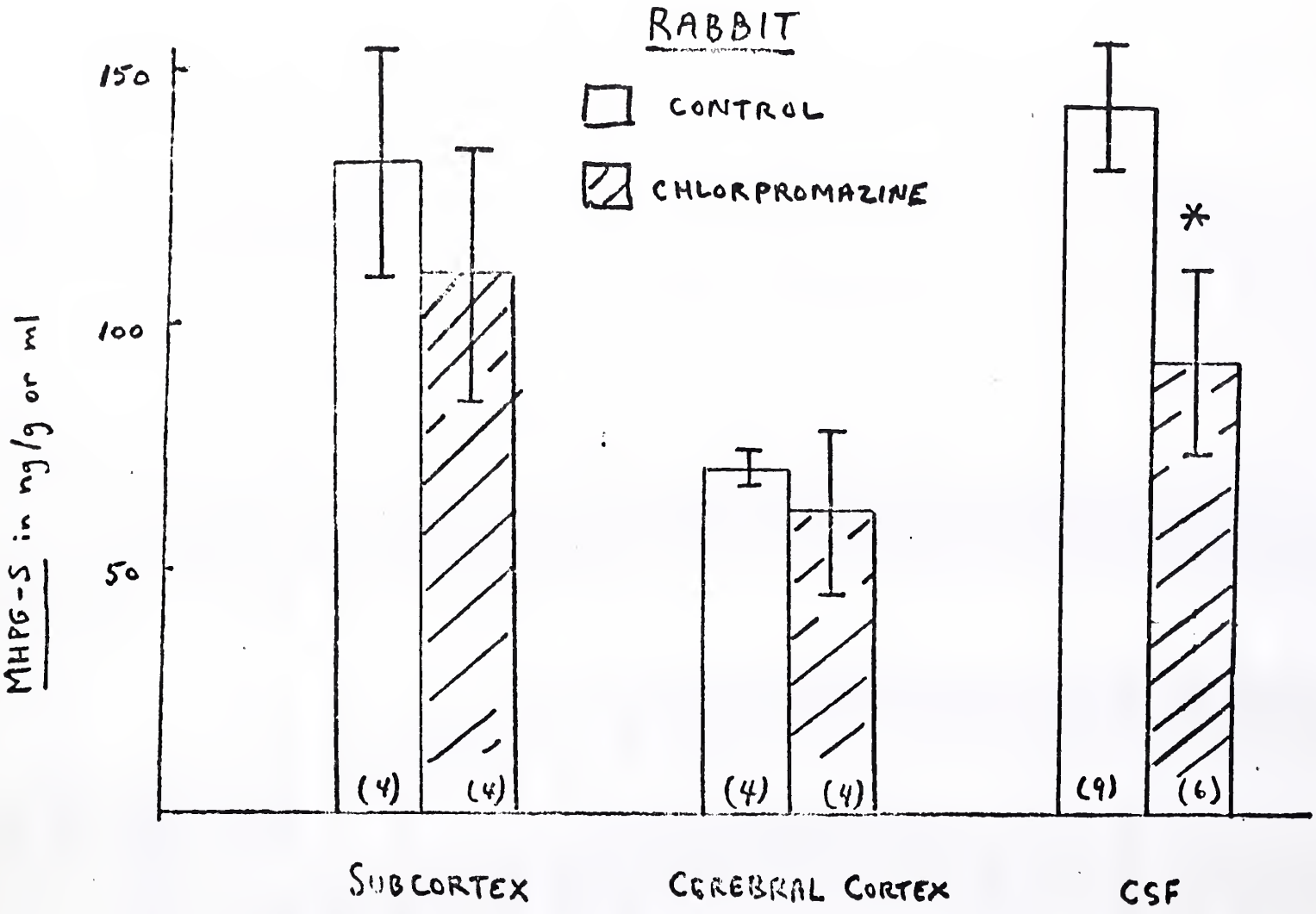

RAT
CONTROL
CHLORPROMAZINE
MHPG-S in ng/g
150
100
50
(8)
(8)
(8)
(8)
*
SUBCORTEX
CEREBRAL CORTEX
*
10,000
5,000
1,000
(4)
(4)
CAUDATE
HVA in ng/g

Figure 10. Levels of 3-methoxy-4-hydroxyphenylglycol-sulfate (MHPG-S) in the brain and cerebrospinal fluid (CSF) of rabbits 4 hours after injection of probenecid (150 mg/kg i.p., repeated every two hours). Some of the rabbits were pretreated with chlorpromazine (10 mg/kg i.v.) 2 hours before the first probenecid injection. Each bar graph represents the mean in ng/g brain or ml CSF $\pm$ S.E.M. for the number of rabbits indicated in parentheses. Significant chlorpromazine-induced changes are noted by (*) for $p < 0.05$ by two-tailed t-test.


RABBIT
CONTROL
CHLORPROMAZINE
MHPG-S in ng/g or ml
150
100
50
(4)
(4)
(4)
(4)
(9)
(6)
*
SUBCORTEX
CEREBRAL CORTEX
CSF

IIIC: MHPG in brain following i.v. ^{3}H-L-dopa---the effect of chlorpromazine

Introduction:

A method for measuring ^{3}H-MHPG formed in brain from systemically administered ^{3}H-L-dopa is presented here, using methods similar to those developed in part I. Exogenous L-dopa is taken up by noradrenergic as well as dopaminergic neurons in the brain (54, 55, 101), and it would be reasonable to assume that if ^{3}H-DA is formed, then ^{3}H-NE is formed as well (see figure 1). If the increased accumulation of ^{3}H-HVA reported in part I reflects increased dopaminergic neuronal activity induced by chlorpromazine, then it would be interesting to see if chlorpromazine has a similar effect on the accumulation of ^{3}H-MHPG. Recent work in rats (79, 80, 81), as well as the results of the foot-shock experiment in rabbits in this thesis seem to imply that MHPG formation in brain reflects brain NE metabolism and neuronal activity.

Methods:

White rabbits were pretreated with the peripheral decarboxylase inhibitor RO4-4602, 50 mg/kg i.p., and one-half hour later were injected i.v. with 100 μCi of ^{3}H-L-dopa as described in part I. Some of the rabbits were pretreated with chlorpromazine, 10 mg/kg i.v., five minutes before the ^{3}H-L-dopa injection. Results were the same in rabbits which were allowed to become hypothermic as in ones whose temperature was maintained. Thus, the results were pooled irrespective of temperature controls. Two or four hours after ^{3}H-L-dopa injection, the rabbits were anesthetized, the

brain removed and dissected as described previously.

The brain tissue was homogenized immediately in 8 ml of 0.4 N perchloric acid and 50 μg of cold MHPG was added to each sample as carrier. The homogenate was centrifuged, and a 2 ml aliquot of the supernatant taken. One ml of 10% barium acetate buffer was added, and each sample was adjusted to pH 5.5 with NaOh. The samples were spun and the supernatant added to 0.4 ml of β-glucuronidase aryl sulfatase (Calbiochem) in order to hydrolyze the sulfate conjugate of MHPG (70). The samples were incubated at 37°C for 20 hours and the raction was then stopped by the addition of 0.2 ml of concentrated perchloric acid.

The pH of each sample was then adjusted to 7.0 with KOH, chilled on ice, and spun to precipitate perchlorate salts. The supernatant was then saturated with NaCl and shaken for 15 minutes with four volumes of nanograde ethyl acetate to extract the MHPG. To the ethyl acetate layer was added 25 μg of cold MHPG and other amine metabolites for future chromatographic visualization. The solution was blown down to dryness with nitrogen, taken up in 100 μl of 80% ethyl alcohol, spun, and 25 μl of the supernatant was spotted on a cellulose thin-layer chromatographic plate for twodimensional chromatographic analysis (49).

The MHPG spot was visualized by spraying with an aniline dye and radioactivity was determined by scintilation counting in 10 ml of triton-100 counting solution. Efficiency was determined by individual ^{3}H-toluene standards.

The counting of theMHPG spots presented several methodological problems. First, autofluorescence from the MHPG-dye complex caused the scintillation counter to record artificially

high counts. We eliminated this problem by cooling the samples for at least 24 hours before counting, and by counting the samples several times to make sure that the counts had stabilized. The second problem was that the counts were very low, often less than twice background. In order to improve accuracy, we counted for a long time---100 minutes. We determined a realistic background by scraping a non-radioactive area of the TLC plate and counting that for 100 minutes.

Results:

Results from five separate experiments were expressed as percentage of control in each experiment and pooled. The pooled results are summarized in figure 10. Chlorpromazine induced a statistically significant decrease in the total amount of ^{3}H-MHPG appearing in rabbit cerebral cortex and subcortex two and four hours following i.v. ^{3}H-L-dopa.

Discussion:

Previous work showed that ^{3}H-L-DA peaks in brain one-half hour after the ^{3}H -L-dopa injection and fell off as the ^{3}H-HVA rose (see Part 1). In preliminary work for this experiment, we noted the presence of ^{3}H-NE in brain at two and four hours in amounts sufficient to have served as precursor for the ^{3}H-MHPG present. This information is consistent with the assumption that ^{3}H-L-dopa has labeled the endogenous brain stores of DA and NE, and that the ^{3}H-MHPG represents physiologically released and metabolized brain ^{3}H-NE.

Although stimulation and lesion studies in the rat (79, 80,

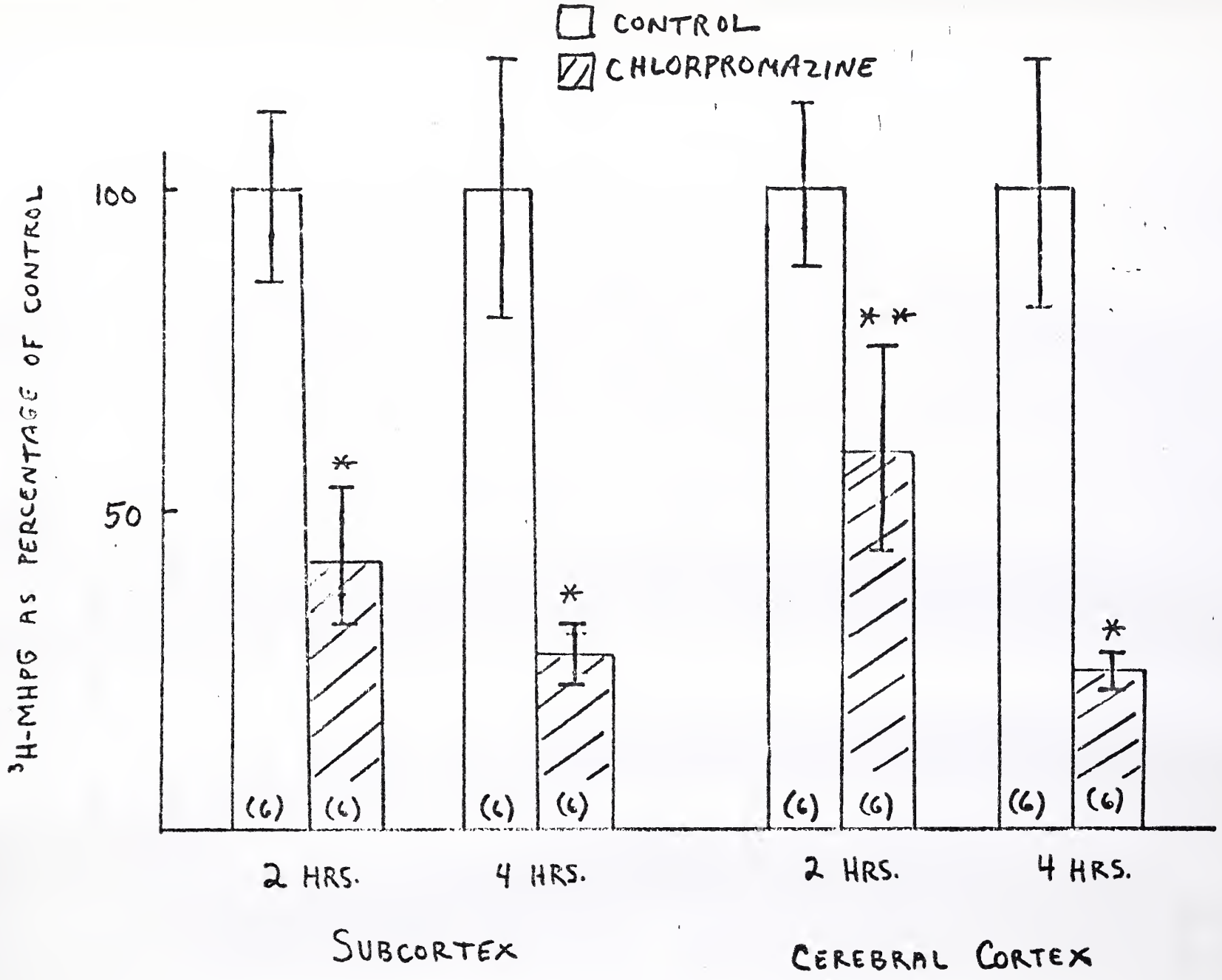
81) showed endogenous MHPG-S to reflect NE neuronal activity in the brain, there is little evidence to prove or disprove that ^{3}H-MHPG formed in brain following i.v. ^{3}H-L-dopa represents activity in NE neurons. Though other neurons besides noradrenergic ones take up exogenous L-dopa (54, 55, 101) and decarboxylate it to DA (82), it is only in neurons which contain the enzyme dopamine beta-hydroxylase that NE and subsequently MHPG could be formed (see figure 1). The distribution of this enzyme seems to follow the distribution of endogenous NE and probably occurs in noradrenergic neurons only (10). On this basis one might expect ^{3}H-MHPG to more accurately reflect noradrenergic neuronal activity than ^{3}H-HVA reflects dopaminergic activity.

The marked decrease in ^{3}H-MHPG measured in brain following chlorpromazine treatment parallels the chlorpromazine-induced decrease in MHPG-S in brain and CSF of the rabbit. As discussed in the previous section, this decrease is not consistent with the small increases in MHPG-S measured in rats (66) and is not consistent with current hypotheses about feed-back activation of NE cells and NE turnover resulting from blockade of post-synaptic NE receptors by chlorpromazine (3). Contrary to the results reported here, chlorpromazine has been shown to enhance the decrease in the specific activity of NE in rats injected with ^{14}C-L-dopa, suggesting an acceleration of the turnover of NE in brain (53). It is difficult to explain how the turnover of labeled NE could increase without increased formation of NE metabolites.

Preliminary results showed that ^{3}H-MHPG was present in CSF but in quantities too small to measure accurately. If the ^{3}H-MHPG in CSF can be shown to represent brain NE metabolism

then perhaps measurement of its accumulation in human CSF following i.v. administration of labeled L-dopa might prove to be a useful index of central noradrenergic function in man.

Figure 11. Radioactivity of total (free plus the sulfate conjugate) 3-methoxy-4-hydroxyphenylglycol (^{3}H-MHPG) in rabbit brain. ^{3}H-MHPG was measured 2 or 4 hours after intravenous administration of ^{3}H-L-dopa to rabbits treated with RO4-4602. Some of the rabbits were pretreated with chlorpromazine, 10 mg/kg i.v., five minutes before the first probenecid injection. Values represent the pooled results of several separate experiments. Bar graphs represent the mean percentage of control $\pm$ S.E.M. for the number of rabbits indicated in parentheses. Significant chlorpromazine-induced decreases are noted by (*) for $p < 0.01$ and by (**) for $p < 0.05$ by the two-tailed t-test.


CONTROL
CHLORPROMAZINE
^{3}H-MHPG AS PERCENTAGE OF CONTROL
100
50
*
**
(6)
2 HRS.
4 HRS.
SUBCORTEX
CEREBRAL CORTEX

Discussion: The role of brain dopamine and norepinephrine in the central actions of chlorpromazine and other anti-psychotic drugs

Chlorpromazine is a phenothiazine with antipsychotic properties (43,92). Along with many other antipsychotic drugs, it is thought to block post-synaptic DA and NE receptors in the central nervous system (3, 31, 67). There are several lines of experimental evidence for the propsed receptor-blocking action of chlorpromazine.

Some recent work (35) has demonstrated a DA-sensitive adenylate cyclase in mammalian brain, whose stimulation by DA is blocked by chlorpromazine. This enzyme may be part of the structure of the hypothesized neuronal membrane "receptors."

Chlorpromazine inhibits apomorphine-induced turning of unilaterally striatomized rats to the operated side. This implies a blockade of DA receptors in the striatum (3). Similarly, chlorpromazine inhibits the L-dopa-induced increases in flexor reflex activity in spinal rats. This implies blockade of NE receptors in the spinal cord (3).

Increased pre-synaptic activity in DA and NE neurons following chlorpromazine and other related antipsychotic drugs has been measured by a variety of methods. This increased activity is thought to be a compensatory feedback response (31) to blockade of post-synaptic receptors, and in animals has been measured directly by single unit recording of individual neurons (28, 29, 61). This increased neuronal activity is also reflected by increased turnover as measured by disappearance of DA and NE following synthesis inhibition (3, 5, 16, 38, 93), by isotopic

labeling techniques (53, 95, 96), and by measuring the levels of DA and NE metabolites in brain (1, 4, 6,28, 31), CSF (20, 25, 64, 100) and urine (27).

Chlorpromazine, like most antipsychotic drugs, seems to block both DA and NE receptors but seems to be more potent in its blockade of the DA receptor. Whereas the rate of firing of dopaminergic neurons in the substantia nigra and ventral tegmental areas is increased following chlorpromazine (28), this same drug has little effect on the firing rate of noradrenergic cells in the locus coeruleus (Walters, J.R., unpublished results, 1973). Chlorpromazine has been reported (61), however, to antagonize amphetamine-induced slowing in these NE cells. Studies of the effect of chlorpromazine on DA and NE turnover have yielded contradictory results.

Using alpha-methyltyrosine, an inhibitor of catecholamine biosynthesis, some investigators (93) have obtained results indicating a selective increase in DA turnover following chlorpromazine. Others (5, 38) have obtained histochemical and biochemical results providing evidence that there is an accelerated disappearance primarily of cerebral NE after alpha-methyltyrosine and chlorpromazine. If the chlorpromazine treatment were continued for 2-3 days, there also appeared an accelerated disappearance of cerebral DA (38). Studies on the accumulation and decline of ^{14}C-DA and ^{14}C-NE formed from exogenously administered ^{14}C-tyrosine precursor have shown that chlorpromazine markedly increases DA synthesis and turnover while increasing NE synthesis at only the highest doses (95, 96).

The results reported in parts I and III of this thesis

reveal a marked difference in the effect of chlorpromazine on the DA and NE systems. Chlorpromazine markedly increased the accumulation in brain and CSF of a major DA metabolite, HVA, as has consistently been reported by others (1, 4, 6, 64). However, as demonstrated in part III and reported by others (72) chlorpromazine had only slight effects on the accumulation of a major Ne metabolite, MHPG-S. If these results indicate a selective increase in brain dopaminergic activity with noradrenergic activity relatively unaffected, then the data are consistent with the hypothesis that chlorpromazine is a more potent blocker of DA receptors than of NE receptors. An alternate interpretation is that chlorpromazine does block post-synaptic NE receptors but the feed-back mechanisms operative in DA systems are less sensitive In NE systems. However, histochemical studies in spinal rats demonstrate that chlorpromazine increases the nerve impulse flow from cell bodies of the bulbospinal neurons (2). Also, other antipsychotic drugs cause more marked elevation of MHPG-S in rat brain (72), as the feed-back hypothesis would predict.

The above and other results have led to the hypothesis that the antipsychotic properties of chlorpromazine and similar drugs are directly related to the ability of these drugs to block DA receptors in the brain (3, 75, 110, 134). In order to verify this hypothesis the ratio of potency of DA to NE receptor blockade should be known for a variety of drugs. The relative potencies of one drug to the other in blocking DA and NE receptors and in clinical antipsychotic activity must be known as well. Either piece of information alone could be misleading.

It is theoretically possible that most antipsychotic drugs

could be more potent in blockade of DA than NE receptors but that antipsychotic potency could still correlate best with the still quite potent blockade of NE receptors. Thus the evidence in this thesis that chlorpromazine in the dose of 10 mg/kg induces a much greater percentage increase in DA metabolites than in NE metabolites could be misleading. In fact, a dose of chlorpromazine six times that of haloperidol has been reported to be necessary to raise MHPG-S in rat brain 25 percent (72). But a dose of chlorpromazine about twelve times that of haloperidol is equipotent in raising HVA levels (1). The clinical antipsychotic potency of haloperidol is forty or fifty times that of chlorpromazine (43). Thus the twelvefold potency difference between the two drugs on raising HVA correlates more closely with the relative clinical potencies than does the six-fold potency difference between the two drugs in raising MHPG-S. If a dose of chlorpromazine 25 times that of haloperidol had been needed to raise MHPG-S levels, then the effect on NE systems would have correlated better with the antipsychotic properties, despite the fact that both drugs seem to block DA receptors more strongly than NE receptors.

Listing the relative potencies of many drugs on only one monoamine system - for example, on their ability to raise MHPG-S - can be misleading too. Thus in the example above, if either haloperidol had been more potent or chlorpromazine had been less potent in its effects on MHPG-S, then this would have been better evidence for the theory that NE receptor blockade is responsible for antipsychotic action. The order of potency can be misleading if not compared to the order of potencies on the DA system and

clinical effect.Absolute dosage must be considered as well. Haloperidol, for example, has been reported to have varying effects on noradrenergic function (3, 5, 38) but is one of the most potent antipsychotic drugs in increasing MHPG-S (72). Yet in the very small doses in which it is clinically useful it has little effect on MHPG-S levels (72).

Although these strict criteria are difficult to fulfill in one experiment, the weight of evidence supports the above hypothesis. In functional, biochemical, and electrophysiological studies in the rat the most potent and specific antispychotic drugs seem to influence mainly brain DA mechanisms (3). It seems unlikely that this correlation is accidental and not causally related. The relative antipsychotic potencies of the various drugs seem to correlate much better with effects on DA turnover and neuronal activity than with effects on NE neuronal systems (3).

Almost all known antipsychotic drugs in high enough doses produce parkinsonian and other extrapyramidal side effects presumably because of their interference with dopaminergic transmission in the striatum (75, 110. 124). The tendency to produce these extrapyramidal effects correlates well with the antipsychotic potencies (43). It has been suggested that the potency of antipsychotic drugs in enhancing NE turnover parallels their sedative properties (72). Thus drugs such as pimozide (19) and haloperidol (43) are reported to be potent antipsychotic drugs likely to cause extrapyramidal effects but with little sedative action. These are potent blockers of DA receptors and markedly enhance DA turnover but have weaker effects on NE systems (1, 3, 4, 5, 28, 72, 95). Drugs such as thioridizine (43)

must be used in much higher doses to be effective antipsychotic agents, are unlikely to cause extrapyramidal effects, and are sedating. This drug blocks both DA and NE receptors when used in relatively high doses (3, 16, 72). Chlorpromazine is a drug which is clinically quite similar to thioridizine (43). It is somewhat surprising that the effect of chlorpromazine on NE turnover has not been more consistently documented. Another interesting antipsychotic drug is clozapin. This compound rarely causes extrapyramidal symptoms but does increase HVA in the striatum and certain limbic area in animal brain (6). It has been suggested that the anticholinergic properties of some antipsychotic drugs are related to the tendency to produce fewer extrapyramidal effects (6).

Though attempts to correlate antipsychotic potencies with effects on DA and NE turnover can become quite confusing, the simple observation that most antipsychotic drugs affect dopaminergic neuronal systems seems clear. Thus it seems now that it is the blockade of DA receptors that is crucial to the antipsychotic effects of these drugs. The site of the antipsychotic action is still unknown, but the distinction between effects on striatal and limbic DA projections is being investigated (4).

Reinforcing the notion that DA and NE activity are involved in the pathogenesis and treatment of psychosis has been the use of amphetamine-induced stereotypy in animals and amphetamine-induced paranoid psychosis in man as models for schizophrenia (7, 8, 44, 45, 108, 110, 124, 125). Amphetamines are known to potentiate the central actions of DA and NE by causing the

release and interfering with the re-uptake of both these catecholamines at their respective synapses (121). There is considerable evidence in animals to support the hypothesis that amphetamine stereotypy is due to increased availability of DA at its post-synaptic receptors in the basal ganglia. Alpha-methyl-paratyrosine blocks synthesis of DA and NE and prevented the induction of stereotyped behavior in the rat by amphetamine (133), while an inhibitor of the conversion of DA to NE did not prevent amphetamine stereotypy (107). Lesions of the corpus striatum prevented the development of the same behavior (109) while direct application of DA to this region elicited the behavior without amphetamine (46).

Based on behavioral and biochemical differences between d- and l-isomers of amphetamine some investigators (8, 42, 124, 125, 129) have suggested that amphetamine psychosis in humans is a result of the potentiation of the effects of brain DA. Other investigators have disputed the differences between isomers which support the above theory (29, 41, 65, 122, 127, 132). Amphetamine psychosis in humans remits readily in response to antipsychotic medication (Angrist, B., unpublished observation).

It is important to keep in mind when discussing schizophrenia that this diagnosis can refer both to an acute psychotic state and to a long-term debilitating process (43). The efficacy of antipsychotic drugs like chlorpromazine is better documented for the former (92) than for the latter. We are just beginning to understand

the interplay of genetic, biological, social, emotional, symbolic, and cognitive functioning which are included in the term " schizophrenia " (103, 126). Certainly the role of the antipsychotic drugs in alleviating the symptoms of schizophrenia must be studied in more detail on a variety of levels.

Perhaps measurement of DA and NE metabolites in human CSF will prove to be a clinically useful method for studying the central metabolism of the catecholamines. The interaction of drugs such as chlorpromazine with brain DA and NE in the therapy of schizophrenia might be better understood using this methodology. As demonstrated in this thesis, accumulation of endogenous metabolites in the CSF, as well as accumulation in the CSF of labeled metabolites formed from radioactive precursor of DA and NE can reflect changes in central dopaminergic and noradrenergic function.

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