Title
The effect of adrenocortical hormones on dopamine B-hydroxylase in regions of rat brain

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THE EFFECT OF ADRENOCORTICAL HORMONES ON DOPAMINE-β-HYDROXYLASE IN REGIONS OF RAT BRAIN

by

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A B S T R A C T

The primary goal of this dissertation work was to investigate changes in the activity of dopamine-β-hydroxylase (DBH) in regions of rat brain under various functional states of adrenocortical system. This constitutes a part of the research trying to elucidate the neural and humoral mechanisms regulating ACTH secretion. It appears that central aminergic neurons modulate the secretion of the hypothalamic releasing and inhibiting factors that control the secretion of the anterior pituitary gland. Recent pharmacological experiments have provided the evidence that a central noradrenergic mechanism inhibits ACTH secretion, presumably via CRF. The question left unanswered is the physiological role of this inhibitory system. One possibility is that the negative feedback effect of glucocorticoids on ACTH secretion is mediated, at least in part, via this noradrenergic system. I studied changes in the activity of DBH in regions of rat brain following manipulation of the adrenocortical system, to see in general whether corticosteroids have an effect on this neurotransmitter synthesizing enzyme in the brain, and to see in particular whether the feedback action of corticosteroids on the central nervous system is at this step.

DBH activity in the hypothalamus (site of noradrenergic endings, and the final common site of neuroendocrine integration), brain stem (site of cell bodies of noradrenergic neurons) and hippocampus (site of noradrenergic endings, and the brain area taking up and concentrating glucocorticoids most effectively) was measured by a radioenzymatic assay. The catecholamine content in those regions was measured by a fluorometric procedure.

The formation of ^{3}H-norepinephrine (^{3}H-NE) in the hypothalamus after injecting ^{3}H-dopamine (^{3}H-DA) into the cerebral ventricle of living rats was determined as an index of in vivo DBH activity.

High levels of DBH were found in the hypothalamus and the brain stem. The anterior half or ventral half of the hypothalamus had higher DBH activity than their corresponding counterparts. There was no circadian fluctuation of DBH activity in the hypothalamus and brain stem of the rat, nor did pentobarbital anesthesia affect the level of DBH in the brain. Shortly after exposure to ether vapor, there was no change in brain DBH activity. When rats were sacrificed 24 hours later, however, DBH activity in the hypothalamus increased. Injection stress and surgical stress, either acutely or chronically, did not change DBH activity in the brain. Although immobilization stress acutely decreased DBH content, repeated immobilization increased DBH activity in the brain.

Four hours after a large dose of corticosterone (10 mg/100 gm B.W.), DBH activity in the hypothalamus increased by 7 %, whereas that in the brain stem and hippocampus remained unchanged. No parallel increase in the static concentrations of norepinephrine and dopamine could be obtained, nor was there any change in the accumulation of ^{3}H-NE in the hypothalamus after ^{3}H-DA was introduced into the third ventricle. When the rat received similar treatment of corticosterone daily for 5 days, DBH activity in the hypothalamus, brain stem and hippocampus all increased by 15-20 %. Norepinephrine and dopamine concentrations in the hypothalamus decreased moderately, although catecholamine concentrations in the brain stem did not show any change.

A 10-20 % decrease in DBH activity in the hypothalamus and brain stem

was observed one to two days after bilateral adrenalectomy; this decrease was not restored by physiological doses of corticosterone. The decrease was not attributable to sodium deficiency or lack of neural connections between the adrenal glands and the central nervous system. In addition, this decrease in DBH activity was not accompanied by any change in catecholamine content. Five days after adrenalectomy DBH activity in the hypothalamus returned to normal, while that in brain stem remained low. Although there was a decrease in DBH activity measured by the *in vitro* technique, the conversion of ^{3}H-DA to ^{3}H-NE in the hypothalamus of living rats was accelerated 2 days after adrenalectomy. This suggests an increased *in vivo* DBH activity.

Thus DBH in specific regions of rat brain is affected by adrenal glands through mechanism(s) other than by glucocorticoids alone. Based on these observations, one can not conclude that the site of glucocorticoid feedback on the central nervous system is in the noradrenergic neurons, or more specifically, whether it is at this enzymatic step. The changes in DBH activity after steroid deficiency or excess are not of great magnitude. Minor changes in DBH activity are not accompanied by parallel changes of catecholamine content or *in vivo* synthesis of norepinephrine. In contrast, the decrease in hypothalamic DBH activity after adrenalectomy is associated with an increase in the dynamic turnover of norepinephrine, manifested by both the increased formation and increased disposition of the neurotransmitter. This finding suggests that DBH in the brain is not rate-limiting for the biosynthesis of norepinephrine, and that a change in the *in vitro* activity of an enzyme in certain tissue is not necessarily accompanied by a parallel change in its *in vivo* activity.

In a study on the biochemical properties of partially purified DBH from beef adrenal gland, it was found that ascorbic acid was essential for the *in vitro* DBH activity. The ascorbic acid could be replaced by a NADH-phenazine methosulfate system which generates superoxide anions, thus suggesting that the role of ascorbate is probably in the generation of superoxide free radicals which are responsible for the activation of the enzyme. It was also demonstrated that hydralazine, an antihypertensive drug, inhibits DBH activity *in vitro* by chelating cupric ions which are required for the neutralization of endogenous DBH inhibitors present in crude enzyme preparations.

A C K N O W L E D G M E N T

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Lastly, I dedicate this accomplishment to my beloved family, particularly to my parents, Mr. and Mrs. Young-Ming Shen.

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R E V I E W O F L I T E R A T U R E

==== C H A P T E R I - 1 ====

STATEMENT OF THE PROBLEMS

Modern pharmacological experiments have provided the evidence that central noradrenergic mechanism affects CRF*-ACTH**-corticosteroid secretion. The physiological significance of this phenomenon, however, remains unknown. One obvious possibility is that the feedback effect of glucocorticoids on CRF-ACTH secretion is mediated, at least in part, via this noradrenergic system.

Dopamine-β-hydroxylase (DBH), the final enzyme catalyzing the biosynthesis of norepinephrine, is uniquely localized in the cell body and endings of noradrenergic neuron. I therefore studied the changes in the activity of DBH in regions of rat brain under various functional states of pituitary-adrenocortical system. I attempted to elucidate in general whether the glucocorticoids have effect on this neurotransmitter synthesizing enzyme in the brain, and in particular whether the proposed feedback action of glucocorticoids on the central nervous system is at this step.

In order to relate the measurement of the <u>in vitro</u> DBH activity to the actual functional capacity of the enzyme in living animal, I measured norepinephrine and dopamine content in brain regions and the formation of ^{3}H-norepinephrine in the hypothalamus after injecting ^{3}H-dopamine into the cerebral ventricles.

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* CRF = Corticotropin releasing factor.

** ACTH = Adrenocorticotropic hormone.

In the course of reviewing the biochemical properties of DBH, it became apparent that the role of some required cofactors had been poorly defined. In a collaborative study I characterized the role of ascorbic acid in this catalytic reaction, and examined the effect of hydralazine, a chelating and antihypertensive agent, on this enzyme.

==== C H A P T E R I - 2 ====

NEURAL REGULATION OF ENDOCRINE SECRETION

The relation between the nervous system and the endocrine system is reciprocal: The brain, largely but not exclusively via the hypothalamus, regulates the secretion of a large portion of the endocrine system, and the hormones secreted by the endocrine glands act back in turn on the brain to modify its function in a variety of ways. In an excellent review Ganong (1966) summarized the neuroendocrine integrating mechanisms which involve the interaction of the nervous system and the endocrine system, thereby regulate a wide variety of body functions and bring about adjustments to meet environmental changes.

An interesting theme in modern neuroendocrine research is the ubiquitous occurrence of adrenergic neurons as regulators of endocrine secretion. This regulation occurs both in glands such as the juxtaglomerular cells, the pancreatic islets and the pineal gland which are innervated by postganglionic sympathetic neurons, and in the hypothalamus, where the cells that secrete the releasing and inhibiting factors that regulate anterior pituitary secretion are apparently innervated by norepinephrine-secreting and dopamine-secreting neurons in the brain. Adrenergic neurons generally do not constitute the sole nor the primary regulator of individual endocrine glands; rather, they modify the response to other stimulatory or inhibitory factors. A recent review by Ganong (1974a) summarized the experimental evidence and documented the fact that the secretion of most and possibly all endocrine glands is influenced, at least in part, by the discharge of noradrenergic and dopaminergic neurons (Table I-1).

TABLE I-1 Catecholaminergic Secretomotor Innervation of the Endocrine System. (Modified from Ganong, 1974a)

Endocrine Gland	Neural Input and Receptor Mechanism*	Stimulatory (+) or Inhibitory (-)
Juxtaglomerular cells in kidney	NA - β	+
Erythrogenin-secreting cells in kidney	NA - β	+
Insulin-secreting cells (β cells)	NA - β NA - α	+ -
Glucagon-secreting cells (α cells)	NA - β NA - α	+ -
Thyroid follicles	NA	+
Parathyroid	NA	+
Pineal	NA - β	+
Paraventricular and supraoptic neurons	NA	-
Cells secreting hypothalamic hormones	NA - α NA - α DA	+ - +

* NA = Noradrenergic; DA = Dopaminergic.
α = Alpha-adrenergic; β = Beta-adrenergic.

It is well known that the secretion of all anterior pituitary hormones is under the control of hypothalamic releasing and inhibiting factors secreted into the portal hypophyseal vessels and transported directly from the hypothalamus to the pituitary gland. There are six generally accepted anterior pituitary hormones, and there is an excitatory hypothalamic hormone for each. In addition, there are hypothalamic factors that inhibit the secretion of prolactin and growth hormone (Fig. I-1).

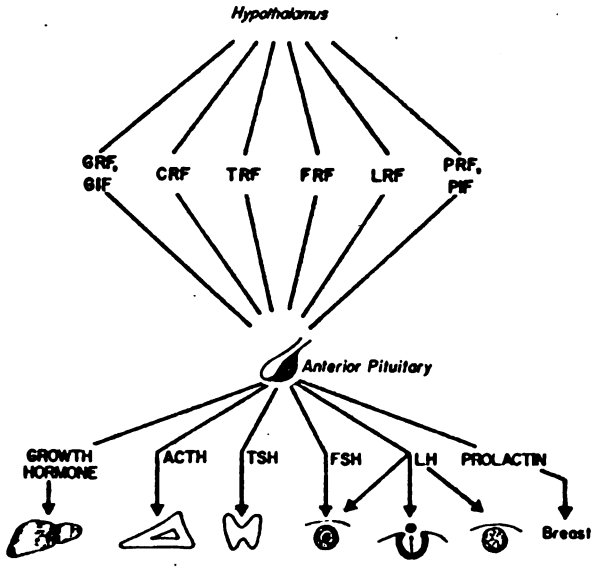


Fig. I-1 Hypothalamic Hormones.

The releasing and inhibiting factors are shown in association with the anterior pituitary hormones they regulate. (From Ganong, 1974a).

GRF = Growth hormone releasing factor
GIF = Growth hormone inhibiting factor
CRF = Corticotropin releasing factor
TRF = Thyrotropin releasing factor
FRF = Follicle stimulating hormone releasing factor
LRF = Luteinizing hormone releasing factor
PRF = Prolactin releasing factor
PIF = Prolactin inhibiting factor

The secretion of the hypothalamic hormones (or factors) is regulated in large part by neural circuits in the hypothalamus and adjacent portions of the brain, and two of the principal transmitters in these circuits are norepinephrine and dopamine. It now appears that there are epinephrine-containing neurons as well. Thus, the secretion of the thyroid, adrenal cortex and gonads as well as the anterior pituitary secretion of growth hormone and prolactin is regulated by portions of the autonomic nervous system in the brain. All the evidence accumulated seems to support the

view that brain catecholamines participate in controlling the secretion of hypophysiotropic hormones from neuroendocrine transducer cells in the hypothalamus (Wurtman, 1971; Ganong, 1974a, 1974b). In brief, the secretion of GRF, FRF, LRF and TRF (full names in the legend of Fig. I-1) is stimulated by noradrenergic mechanism in the hypothalamus, probably alpha-mediated. The situation for PIF is unique, in that dopamine is the excitatory transmitter for its release. Currently there is evidence indicating that dopamine itself may well be the PIF (Shaar and Clemens, 1974). Lastly, the experimental results relating a possible role of catecholamines in the regulation of CRF release are somewhat conflicting. This particular subject will be discussed in the next chapter.

==== C H A P T E R I - 3 ====

BRAIN CATECHOLAMINES AND CRF-ACTH SECRETION

The central nervous system (CNS) plays an essential role in the regulation of synthesis and release of ACTH. The hypothalamus is established as the focal point at which neural stimuli converge to influence the secretion of ACTH and the median eminence is regarded as the final common path through which information is transmitted to the anterior pituitary. Although a corticotropin releasing factor (CRF) has not yet been chemically identified and synthesized, the neurovascular hypothesis of anterior pituitary hormone regulation appears to be well established. Release of CRF and subsequent stimulation of ACTH secretion is dependent on a variety of factors. First, a diurnal rhythm in ACTH secretion is maintained by a biological clock in certain part(s) of the brain. Second, the corticosteroid-ACTH-CRF interactions constitute closed loop feedback mechanisms that operate to stabilize the rate of ACTH secretion. Third, excitatory neural inputs converge on the hypothalamus and represent open loop components providing a mechanism by which feedback control can be overridden to bring about increased ACTH secretion in response to stress. Fourth, an inhibitory component may possibly provide some control over the ACTH secretion.

The morphological substrate for the mediation of many of these factors includes neural networks which involve many brain areas and are integrated in the hypothalamus. The neurotransmitters in these pathways include acetylcholine and three monoamines, norepinephrine, dopamine and serotonin. All of them have been implicated in some way in the regulation of ACTH secretion. There is evidence that acetylcholine acts as an excitatory

transmitter for the ACTH secretion (Endröczi et al., 1963; Krieger and Krieger, 1965; Hedge and Smelik, 1968), and a considerable literature exists that suggests a role for serotonin in the regulation of ACTH secretion (Naumenko, 1968; Krieger and Rizzo, 1969; Vernikos-Danellis et al., 1973). However, this thesis is concerned with the relationships between catecholamines and ACTH secretion. Van Loon (1973) has written a comprehensive review on this subject.

Although some data indicate no correlation between brain catecholamines and ACTH secretion (Smelik, 1967; Smelik and Bilt, 1967; Carr and Moore, 1968; Hirsch and Moore, 1968; Kumeda et al., 1974), most studies have shown some sort of correlation.

Excitatory Role of Brain Catecholamines. Several investigators have demonstrated release of ACTH following injection of catecholamines directly into the brain. Epinephrine, norepinephrine and ephedrine injected into the posterior hypothalamus or ventral tegmentum increased adrenal venous corticosteroid output in cats (Endröczi et al., 1963). This adrenergic stimulation of ACTH secretion was confirmed again in cats by Krieger and Krieger (1965) who reported that norepinephrine injected into the median eminence or mammillary bodies produced increased levels of plasma 17-hydroxycorticosteroids. Subsequently these authors reported that implantation of norepinephrine in the median eminence, midportion of the mammillary body, lateral and basal amygdala, dorsal septal area and hippocampal formation was associated with an abrupt increase in plasma 11-hydroxycorticosteroid levels (Krieger and Krieger, 1970). Naumenko (1968) confirmed the findings of Endröczi et al. (1963) in guinea pigs with the demonstration that norepinephrine administrated directly into the posterior hypothalamus or dorsal midbrain increased plasma 17-OHCS.

Inhibitory Role of Brain Catecholamines. Inhibitory neural structures in the brain regulating ACTH secretion have been reviewed (Mangili et al., 1966). Egdahl (1960) demonstrated that removal of the brain leaving an isolated hypothalamus elevates 17-OHCS secretion in the dog. Deafferentation of the hypothalamus in the rat by cutting all connections with a stereotaxic knife leads to elevated levels of corticosteroid secretion (Halász et al., 1967; Halász, 1969). The basal forebrain region appears to form part of the inhibitory input to the hypothalamus. Electrical stimulation of this region resulted in decreased cortisol secretion in cats (Taylor and Branch, 1971). In addition, stimulation of certain parts of limbic structures, the hippocampus in particular, has been reported to decrease the resting plasma corticosteroid levels in monkey (Mason, 1958) and man (Mandell et al., 1963; Rubin et al., 1966), and to inhibit the adrenal stress response in cats and rabbits (Endröczi and Lissak, 1962; Kawakami et al., 1968). Thus neural mechanisms mediating inhibition of ACTH secretion have been demonstrated. Several catecholaminergic pathways in the brain have been described recently (Ungerstedt, 1971), and some of them may be implicated as part of the neural substrate inhibiting ACTH secretion.

There have been scattered reports for a number of years of pharmacological experiments that suggest the central adrenergic mechanism inhibiting ACTH secretion in resting state and in response to stress. Most of them were done with drugs that affect the metabolism and functional state of central catecholaminergic neurons (for references, see Van Loon, 1973). Recently, Ganong and his associates have carried out studies which establish the relation of brain adrenergic systems to the regulation of ACTH secretion in the dog and the rat. Their evidence fits best with the hypothesis that a central neural system inhibits ACTH secretion, that

norepinephrine is the mediator in that system, and that its effects are mediated by an alpha-adrenergic mechanism. The inhibitory effect is probably exerted directly on the cells that secrete the hypothalamic factor, CRF (for review and references, see Ganong, 1972a, 1972b, 1973, 1974b). One interesting feature of the changes in ACTH secretion produced by this system is that they are apparently transient, rather than prolonged.

Although the work by Ganong and associates has provided considerable evidence in support of the concept of central noradrenergic inhibition of ACTH secretion, the physiological significance of this inhibition is unknown. An intriguing possibility is that it mediates in part the known inhibitory effect of glucocorticoids on CRF-ACTH secretion. It has been established that glucocorticoids feed back at the level of pituitary, and there is additional evidence that they also feed back at the brain level as well (Mangili et al., 1966; Ganong, 1970). Dallman and Yates (1968) have reported that the monoamine oxidase inhibitor iproniazid potentiates the ACTH-inhibiting effect of the synthetic glucocorticoid, dexamethasone, an observation which has been confirmed afterwards (Ganong, 1972b). Nicolescu-Catargi et al. (1970) have reported that monoamine oxidase inhibitors restore the ability of dexamethasone to inhibit ACTH secretion in patients with Cushing's disease. These findings suggest that brain amines may be involved in steroid feedback.

To explore the possibility that glucocorticoids inhibit ACTH secretion in part via an action on a central noradrenergic system, I studied the effects of glucocorticoid excess and deficiency upon the activity of the marker enzyme for noradrenergic neuron, dopamine-β-hydroxylase. I have also studied catecholamine content and <u>in vivo</u> synthesis of norepinephrine

in regions of rat brain. If adrenal corticosteroids do act via a central noradrenergic system, they might exert their effect by changing those biochemical parameters. Furthermore, glucocorticoids affect the enzymes involved in the biosynthesis of norepinephrine in the peripheral nervous tissues (Weinshilboum and Axelrod, 1970; Gewirtz et al., 1971; Keen and McLean, 1974). Therefore, my research casts light on the general physiological question of whether the glucocorticoids have effect on central as well as peripheral noradrenergic neurons.

==== C H A P T E R I - 4 ====

NORADRENERGIC PATHWAYS IN RAT BRAIN

The presence of an adrenergic substance "sympathin" in extracts of mammalian brain was first described by von Euler in 1946. A few years later, Holtz (1950) confirmed this observation in brain and spinal cord and showed that norepinephrine (NE) concentrations far exceed those of epinephrine. Vogt (1954) first demonstrated the uneven distribution of norepinephrine in cat and dog brain. There is a small amount of epinephrine in the mammalian brain. In various studies, its concentration was estimated to range from 5 to 10 % of the total norepinephrine and epinephrine level in the brain (for references, see Hökfelt et al., 1974). Serotonin (5-hydroxytryptamine, 5-HT) was found in brain tissues in 1954 by Amin et al. The presence of dopamine in brain tissue was suspected in 1957 by Montagu, demonstrated that year by Weil-Malherbe and Bone, and confirmed by Carlsson et al. (1958).

The formaldehyde fluorescence method (Falck and Hillarp technique) provided the first proof of a cellular localization of the monoamines (Falck and Hillarp, 1959; Falck, 1962; Falck et al., 1962). Subsequent work described in detail the localization of the noradrenergic, dopaminergic and serotoninergic cell bodies and terminals (for references, see Ungerstedt, 1971). In the central nervous system their distribution has been extensively studied, and there has always been a good correlation between histochemical and biochemical estimations of brain norepinephrine, dopamine and serotonine levels in various regions (Carlsson et al., 1962; Fuxe et al., 1970a; Ungerstedt, 1971). In order to determine the course

of the different monoaminergic pathways, lesions were combined with the fluorescence histochemical method and it was possible to describe the existence of descending noradrenergic (NA) and serotoninergic pathways to the spinal cord and ascending noradrenergic and serotoninergic pathways from the brain stem entering the medial forebrain bundle. The existence of a nigro-striatal, a meso-limbic and a tubero-infundibular dopaminergic systems was proven in a number of studies combining biochemical and histochemical techniques (Ungerstedt, 1971).

It has been possible to separate the ascending noradrenergic pathways into a dorsal and a ventral bundle of axons. The dorsal bundle innervates the whole cerebral cortex and the hippocampus, and the ventral bundle supplies NA terminals to the medulla, the pons, the mesencephalon and the diencephalon. The dorsal bundle is found to originate primarily in the locus coeruleus of the upper brain stem. Lesions of this region abolish the NA nerve terminals in all cortical areas and in several other areas of the brain. The locus coeruleus also sends NA axons to the cerebellum. The diffuse cell groups in the medulla oblongata and the pons, collectively known as locus subcoeruleus, as well as the locus coeruleus, give rise to the ventral ascending noradrenergic bundle. The axons, only partially crossed, ascend in the mid-reticular formation, turn ventro-medially along the lemniscus medialis and continue rostrally mainly within the medial forebrain bundle. This system gives rise to noradrenergic nerve terminals in the lower brain stem, the mesencephalon and the diencephalon. In the mesencephalon, the ventral pathway has been traced to the major terminal areas, i.e. the ventro-lateral part of the substantia grisea centralis and the part of mesencephalic reticular formation dorsal and dorso-lateral to the lemniscus medialis at the caudal

level of the interpeduncular nucleus. The ventral pathway innervates the whole hypothalamus, most notably the dorsomedial nucleus, the periventricular nucleus, the area ventral to the fornix, the arcuate nucleus and the internal layer of the median eminence, the retrochiasmatic area, paraventricular nucleus and supraoptic nucleus, and the preoptic area. Further rostrally the ventral pathway supplies the terminals in the densely innervated ventral part of the nucleus interstitialis striae terminalis.

Thus for different regions of rat brain, the hypothalamus contains noradrenergic terminals from cell bodies in diffuse areas of the brain stem via ventral NA bundle, the hippocampus contains noradrenergic terminals from cell bodies in the locus coeruleus via dorsal bundle, and in the brain stem there are many NA cell bodies as well as some NA axons and terminals.

The role of the ascending noradrenergic pathways in the control of ACTH secretion has been evaluated by Fuxe et al. (1973b) with the help of selective lesions of the various nerve tracts and measurements of plasma corticosterone levels, and with the help of measurements of amine turnover in relation to changes in pituitary-adrenal activity. It is suggested that the ventral noradrenergic bundle to the hypothalamus appears to be an inhibitory pathway with regard to CRF-ACTH secretion, particularly stress-induced hypersecretion of ACTH and may mediate part of the inhibitory feedback of corticosteroids and ACTH on CRF secretion in normal rats.

==== C H A P T E R I - 5 ====

METABOLISM OF NOREPINEPHRINE IN THE CENTRAL NERVOUS SYSTEM

Until 1954, it was generally suspected that brain norepinephrine (NE) was located mainly in blood vessels. In that year, however, Vogt measured catecholamines of cat and dog brain by bioassay and first demonstrated their relatively high concentrations in specific anatomical areas. This regional localization supported the concept, now widely accepted, that catecholamines are located in central neurons and many other experiments have indicated that they act as neurotransmitters at some central synapses.

Whole brain concentrations of norepinephrine have been reported to range from 0.10 to 0.50 µg/gm in several species (for references, see Glowinski and Baldessarini, 1966). Brain norepinephrine concentration appears to increase with age, but a difference in concentration between newborn and adult animals is found only in those species which are neurologically relatively immature at birth, such as the rat and rabbit. The pattern of distribution of norepinephrine first described by Vogt in 1954 for the cat and dog brain is generally similar in other species. The highest concentrations are in the hypothalamus, with a range of 1 to 3 µg/gm in man (Bertler and Rosengren, 1959a; Bertler, 1961), monkey (Pscheidt and Himwich, 1963), cat (Bertler and Rosengren, 1959b; McGeer et al., 1963) and rat (Glowinski and Iversen, 1966). Other regions can be ranked as follows : midbrain and pons, medulla oblongata and striatum, followed by a group of structures of rather low concentration, including hippocampus, cerebral cortex, cerebellum and spinal cord (see Glowinski

and Baldessarini, 1966). There is generally more norepinephrine in gray than in white matter.

Several lines of evidence support the conclusion that amines are contained within specific neurons in the central nervous system. It is now established that norepinephrine is localized primarily in the soma, axon, varicosities and endings of the noradrenergic neuron, and dopamine is present both in the specific dopaminergic neuron as the transmitter, and in the noradrenergic neuron as the immediate precursor for the synthesis of norepinephrine. Although there is evidence for the existence of epinephrine-containing neurons in mammalian brain (Hökfelt et al., 1974) which may containing norepinephrine as precursor for epinephrine, their number is not high. Consequently, the amount of norepinephrine in those neurons is probably negligible.

The entry into the mammalian brain of norepinephrine and its precursor dopamine is severely limited by a blood-brain barrier, which appears to be fully developed at birth in the rat. Nevertheless, these amines are present in the brain in significant concentration; this suggests that they are synthesized locally in brain tissue. Precursors and enzymes for norepinephrine synthesis, first shown in the peripheral sympathetic nervous tissues, are present in the brain.

Biosynthesis of Catecholamines. Brain catecholamines may be synthesized following uptake of either tyrosine or l-dopa (l-3,4-dihydroxyphenylalanine) into the brain; these precursor amino acids appear to be taken up into brain cells by a transport system specifically for neutral amino acids (Wurtman and Fernstrom, 1972). Normally, phenylalanine and tyrosine are present in the brain tissue in low concentrations. Only

in catecholaminergic neurons is l-tyrosine converted to l-dopa. This reaction is catalyzed by tyrosine hydroxylase (Udenfriend, 1966), an enzyme associated with particles as well as the supernatant fraction of the adrenergic tissue homogenate (for references, see Moore and Dominic, 1971). It is localized in all catecholaminergic neurons, i.e. dopaminergic, noradrenergic and epinephrine-containing neurons. The conversion of tyrosine to dopa is the rate-limiting step in the synthesis of norepinephrine in the peripheral sympathetic system (Levitt et al., 1965), and it is likely to be rate-limiting in the brain as well. The best inhibitor of tyrosine hydroxylase is alpha-methyl-para-tyrosine (Spector et al., 1965), which causes a gradual decline in the concentration of dopamine and norepinephrine in the brain.

The second step in catecholamine biosynthesis is the conversion of dopa to dopamine, catalyzed by a rather nonspecific enzyme referred to as dopa decarboxylase or perhaps more appropriately aromatic l-amino acid decarboxylase (Holtz, 1959). Thus the formation of several other amines from decarboxylation of amino acids can occur throughout the brain (Lovenberg et al., 1962).

In the dopaminergic neuron, the biosynthetic pathway terminates here with the formation of dopamine as the final product. In noradrenergic and epinephrine-containing neurons, however, there is a specific vesicle-bound enzyme, dopamine-β-hydroxylase (DBH), which converts dopamine to norepinephrine (Kaufman and Friedman, 1965). This is the last step for norepinephrine synthesis. Under normal and resting state the amount of this enzyme in the neuron is in excess of what required for optimal catalytic reaction, and it appears that several phenylethylamine derivatives can be β-hydroxylated by this same enzyme (Levin and Kaufman, 1961;

Creveling et al., 1962). Many inhibitors of DBH are effective both in vitro and in vivo (Creveling et al., 1962; Green, 1964; Goldstein et al., 1964; Collins, 1965; Johnson et al., 1969; Nagatsu et al., 1970). Some of these agents have been shown in high doses to cause a selective and marked depletion of norepinephrine in central noradrenergic neurons, associated with a small rise of dopamine levels in the brain (Carlsson et al., 1966; Goldstein, 1966; Johnson et al., 1970). Recently, a more potent inhibitor of this category, FLA 63, has been introduced (Svensson and Waldeck, 1969; Corrodi et al., 1970).

The activity of DBH, measured by a radioenzymatic assay (Molinoff et al., 1971), has been shown to present in various chromaffin tissues, sympathetically innervated structures and regions of the brain. Based on the fact that there are few epinephrine-containing neurons in mammalian brain, we can assume that dopamine-β-hydroxylase is primarily localized in the noradrenergic neurons and can be a specific marker for that particular neuronal system. Some special features of the enzyme, along with the specific characteristics of the catalytic reaction, make dopamine-β-hydroxylase occupy another regulatory step for the biosynthesis of norepinephrine (see Chapter I-7). Consequently, in exploring changes in the functional state of noradrenergic neurons after various experimental procedures, I chose this enzyme for my study.

Release of Catecholamines. The mechanism whereby catecholamines are released from the adrenal medulla and peripheral sympathetic neurons involves a process known as exocytosis (Smith and Winkler, 1972). Uopn electrical or chemical stimulation followed by membrane depolarization, the granular vesicles in adrenal medullary cells or nerve endings discharge not only catecholamines but also their entire soluble contents, including

ATP and specific proteins (chromogranins and DBH). The proteins are either metabolized locally or gain access to the general circulation to be metabolized elsewhere. In the central nervous system, this release mechanism for neurotransmitters has not been clearly demonstrated. Nonetheless, it has been generally assumed that the process of catecholamine release in the CNS is similar to that described for the peripheral sympathetic system (Axelrod and Weinshilboum, 1972).

Catabolism of Catecholamines in the Brain. Degradation of catecholamines is dependent mainly on two enzymes, monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT). It appears that the former enzyme is primarily responsible for intraneuronal and the latter enzyme for extraneuronal degradation of catecholamines. The two enzymatic pathways of catecholamine metabolism in the peripheral sympathetic system also occur in the central nervous system (Mannarino et al., 1963; Matsuoka, 1964; Glowinski et al., 1965). When the nerve is depolarized, the granular vesicles in the nerve endings discharge norepinephrine and the soluble portion of DBH into the synaptic cleft by a process of exocytosis as described above. Norepinephrine acts at the effector cell, and its actions are terminated by reuptake into the neuron, removal by circulation and subsequent metabolism in the liver or by metabolism locally in the effector cell by COMT and mitochondrial MAO. Norepinephrine that leaks out of the storage vesicles is inactivated by intraneuronal MAO. The catecholamines are almost entirely metabolized in the body, and only small quantities of unchanged amines are found in the urine. The main metabolites of norepinephrine and of epinephrine in the urine are vanillylmandelic acid (VMA) and 3-methoxy-4-hydroxyphenylethyleneglycol (MOPEG). These compounds result from the action of both MAO and COMT. Maas and

Landis (1968), and LaBrosse (1970) have shown that MOPEG is further metabolized to VMA in peripheral tissues. Thus the MOPEG found in the urine can hardly be regarded as an index of total MOPEG production.

During the past 10 years evidence has accrued to show that the major metabolites of norepinephrine in the brain are the neutral alcohols, MOPEG and DOPEG (3,4-dihydroxyphenylethyleneglycol), in contrast to the acidic product of peripheral norepinephrine metabolism, VMA. Interestingly, the main metabolites for dopamine are acidic for both peripheral and central nervous system; they are primarily homovanillic acid (HVA) for both systems and, to less extent, 3,4-dihydroxyphenylacetic acid (DOPAC) as well for the CNS (Braestrup et al., 1974). The formation of MOPEG was demonstrated in cat brain in vivo by Mannarino et al. (1963) and in rabbit in vitro by Rutledge and Jonason (1967). In 1968, Schanberg et al. (1968a) found that in the rat brain the major metabolite found from radioactively labeled normetanephrine (NM) was the ethereal sulphate conjugate of MOPEG. The presence of MOPEG-sulphate in the brain of some other mammalian species in addition to the free compound was demonstrated again by Schanberg et al. (1968b), who also showed that MOPEG and its sulphate conjugate were present in human cerebrospinal fluid. A new method combining ion-exchange and thin-layer chromatography has recently been described which separated and measured quantitatively, after intraventricular injection of ^{3}H-dopamine, the rat brain content of labeled norepinephrine and its metabolites (Braestrup et al., 1974). The time-course study performed from 5 minutes to 24 hours after ^{3}H-dopamine confirmed that in rat brain MOPEG and to less extent DOPEG, mainly as sulphate conjugates, are major norepinephrine metabolites whereas VMA is a rather insignificant norepinephrine metabolite.

Thus, in studying the functional state of noradrenergic neurons in the brain, several biochemical methods can be used. It has been possible to measure the static concentration of norepinephrine and dopamine, as well as their metabolites, in regions of animal brain by fluorometric methods (Taylor and Laverty, 1969a; Anton and Sayre, 1972) and by a recently developed radioenzymatic assay (Coyle and Henry, 1973). It is also possible to measure the activity of catecholamine synthesizing and catabolizing enzymes in brain homogenate by radioenzymatic procedures (Goldstein, 1972; Jarrott, 1974). Moreover, a wide variety of turnover studies have been adopted to assess the dynamic picture of norepinephrine formation and disposition (Neff, 1972; Weiner, 1974a). They may be divided into "steady state" and "nonsteady state" techniques. Each has particular virtues and, unfortunately, some inherent theoretical defects. With all of the commonly employed methods for estimating biogenic amine synthesis and turnover in vivo, there are either real or potential shortcomings that can not be excluded. The methods owe their popularity to the ease with which most of the procedures can be performed and because they have been found to be empirically useful. In spite of their inherent problems, the methods all seem to provide an estimate of relative rates of catecholamine synthesis and turnover when two or more experimental situations are being compared. I have adopted the one for estimating the formation of norepinephrine in the hypothalamus by injecting tritiated dopamine into the cerebral ventricles, and the other one for estimating the disappearance of norepinephrine by injecting tritiated norepinephrine into the ventricle and following its decline.

==== C H A P T E R I - 6 ====

PROPERTIES OF DOPAMINE-β-HYDROXYLASE

As it has been stated in the last chapter, dopamine-β-hydroxylase (DBH) plays an important role in neurotransmission in the sympathetic neurons because of its unique localization in noradrenergic granules and the reaction it catalyzes. This enzyme, 3,4-dihydroxyphenylethylamine, ascorbate : oxygen oxidoreductase (hydroxylating) or EC 1.14.2.1, hydroxylates dopamine on the beta carbon to form norepinephrine. It has recently been purified, measured by sensitive and specific assays and its properties have been studied in detail (for review, see Kaufman and Friedman, 1965; Goldstein, 1972; Axelrod, 1972; Molinoff and Orcutt, 1973; Geffen, 1974).

The conversion of dopamine to norepinephrine has been demonstrated in vitro with the use of adrenal slices (Hagan, 1956), homogenates (Goodall and Kirshner, 1957) and aqueous extracts of acetone powders (Neri et al., 1956) of adrenal glands. In 1956, Leeper and Udenfriend demonstrated in vivo that when ^{14}C-dopamine was administered to rats, ^{14}C-norepinephrine could be isolated from the adrenal gland. Kirshner (1957) subsequently showed that the enzyme responsible for the conversion of dopamine to norepinephrine was contained in the particulate fraction of adrenal homogenates. It has been well established that DBH is intimately associated with the catecholamine-containing granules and that many of its properties in vivo are determined by the properties of these particles. In view of the presence of norepinephrine-containing vesicles in all tissues with sympathetic innervation so far studied, as well as the brain, it is not surprising that these organs also appear to have the capacity to synthesize

norepinephrine from its precursor amino acids. Goodall and Kirshner (1958) were able to repeat their earlier work with adrenal medullary slices using canine and bovine sympathetic nerves and ganglia. Both ^{14}C-tyrosine and ^{14}C-dopa were converted to norepinephrine. Udenfriend and Creveling (1959) demonstrated the conversion of ^{14}C-dopamine to ^{14}C-norepinephrine in brain homogenates of the rat, dog, cow and sheep. The isolated perfused heart of the rabbit (Musacchio and Goldstein, 1963), dog (Chidsey et al., 1963, 1964) or guinea pig (Spector et al., 1963) also can synthesize norepinephrine from tyrosine.

In 1960, the solubilization and partial purification of dopamine-β-hydroxylase from bovine adrenal medullary tissue was first achieved by Levin et al. Since then, several modifications of the original purification procedures have been published (Friedman and Kaufman, 1965b; Goldstein et al., 1965). Recently, an attempt has been made to further purify dopamine-β-hydroxylase from the chromaffin granule preparation of bovine adrenal medulla (Foldes et al., 1972).

When the enzyme was obtained in a highly purified state, it was estimated to catalyze the hydroxylation of about 1,000 moles of dopamine per mole of enzyme per minute at 25°C (Friedman and Kaufman, 1965a). The enzyme is a copper-containing protein (Friedman and Kaufman, 1965b; Goldstein et al., 1965; Blumberg et al., 1965), with a molecular weight of about 290,000 (Friedman and Kaufman, 1965b). It has been tentatively characterized as a tetrameric glycoprotein with one or two active sites, each containing one copper molecule that undergoes cyclic oxidation and reduction (Wallace et al., 1973). Earlier, Friedman and Kaufman (1965b) have shown that approximately 2 moles of cupric ions (Cu^{++}) are associated with one mole of enzyme, and a variable amount of cuprous ions (Cu^{+}) is

also present. Indeed, it has been implied that only the cuprous form, other than the cupric form, is catalytically active (Friedman and Kaufman, 1965b). Under dissociating conditions, four subunits of molecular weight 75,000 have been reported (Craine et al., 1973), but it is uncertain whether the units are identical or catalytically active. Enzymatic activity may depend upon the co-operative action of two or even all four subunits, and upon sugar residues that comprise 4 % of the molecule (Wallace et al., 1973). Amino acid analysis has revealed that DBH is a highly acidic protein, rich in glutamate and aspartate, and low in methionine and half-cystine (Foldes et al., 1973).

The hydroxylation reaction catalyzed by DBH is aerobic in type, requiring molecular oxygen and an external electron donor. Enzymes which show these characteristics have been classified by Mason (1957) as "mixed function oxidase". The requirement for oxygen is specific, no reaction occurring anaerobically in the presence of other oxidizing agents such as oxidation-reduction dyes. In those systems in which the reaction has been studied with ^{18}O, it has been shown that the hydroxyl-oxygen atom is derived from atmospheric oxygen. The general equation that describes aerobic hydroxylation reaction is

$$RH + O_2 + 2H^+ + 2e^- \longrightarrow ROH + H_2O \ .$$

These criteria serve to distinguish this type of hydroxylation from the more familiar, anaerobic type, which may be illustrated by the conversion of succinate to malate. This kind of hydroxylation is actually a hydration reaction in which the hydroxyl-oxygen is derived from water :

$$-CH_2-CH_2- + A \longrightarrow -CH=CH- + AH_2$$
$$-CH=CH- + H_2O \longrightarrow -CHOH-CH_2- \ ,$$

where A is the hydrogen acceptor. This kind of hydroxylation reaction may also need oxygen to reoxidize the reduced form of the hydrogen acceptor. Therefore, the mere demonstration that a hydroxylation reaction is dependent on oxygen is not sufficient evidence for classifying it as an anaerobic hydroxylation reaction. It is apparent that the two types of hydroxylation reaction can be distinguished by their requirements - an electron acceptor for the hydration type and an electron donor and oxygen for the aerobic type.

The first indication that dopamine-β-hydroxylase is a mixed function oxidase was the demonstration of a requirement for ascorbate (Levin et al., 1960). It was found that this reducing agent could stimulate the hydroxylation reaction even in adrenal particles, and the requirement for ascorbate became more pronounced as the enzyme was purified. With the identification of ascorbate as an essential component of the hydroxylating system, it was possible to determine the stoichiometry of the reaction. It was found that equimolar amounts of dopamine, oxygen and ascorbate were consumed and an equivalent amount of norepinephrine was produced (Levin et al., 1960). From these results, the hydroxylation reaction was formulated as :

$$\text{Dopamine} + \text{ascorbate} + O_2 \longrightarrow \text{L-norepinephrine} + \text{dehydroascorbate} + H_2O \ .$$

The specificity of the ascorbate requirement has been studied (Levin et al., 1960). Reduced pteridines were shown to have only slight activity. It is significant that the two hydroxylation steps in the pathway for the biosynthesis of norepinephrine utilize different electron-donating cofactors ; the first step in the pathway, the ring-hydroxylation of tyrosine, involves a tetrahydropteridine, whereas the side-chain hydroxylation of dopamine involves ascorbate. Moreover, there is essentially no overlap

in specificity; ascorbate is inactive in the tyrosine hydroxylating system and tetrahydropteridines have only slight activity in the dopamine-hydroxylating system (Kaufman and Friedman, 1965). Whether or not ascorbate functions as a cofactor for this enzyme in vivo is still uncertain. It has been shown that an enzyme is present in animal tissues that can catalyze the conversion of dehydroascorbate to ascorbate (Hughes, 1964). This enzyme would amplify the ability of small amounts of ascorbate to participate in the hydroxylation reaction. When large doses of ascorbic acid were given to intact rats (500 mg/kg, intraperitoneally), a decrease of dopamine content and an increase of norepinephrine content in different cerebral structures were provoked after 20 minutes (Izquierdo et al., 1968). It is possible that ascorbate indeed act in vivo as a cofactor to activate the hydroxylating enzyme.

Since DBH is a copper enzyme, various chelating agents can effectively inhibit the enzyme activity in vitro and in vivo (see Chapter I-5, p. 19; Green, 1964; Goldstein et al., 1964). In view of the effect of chelating agents on DBH activity, I tested hydralazine on the DBH activity in vitro. Hydralazine, a chelating agent, has been used to treat certain patients with high blood pressure; however, little is known at the present time about the precise mechanism(s) of its antihypertensive action. It would be expected that hydralazine inhibits DBH in vitro by chelating cupric ions which are required for enzyme activity. The enzyme is also inhibited by a large group of substrate analogs (Creveling et al., 1962; Goldstein and Contrera, 1962).

In an attempt to gain some insight into the mechanism of the hydroxylation reaction, Friedman and Kaufman (1965b) were able to establish the sequence in which ascorbate, oxygen and dopamine interact with the

enzyme. With the availability of relatively large amounts of highly purified enzyme, a separation of the hydroxylation reaction into two partial reactions has been accomplished. These reactions are shown below:

$$E + \text{ascorbate} \longrightarrow E^{=} + \text{dehydroascorbate} + 2H^{+}$$

$$E^{=} + O_2 + RH + 2H^{+} \longrightarrow E + ROH + H_2O ,$$

where E stands for enzyme, $E^{=}$ for a reduced form of enzyme, and RH for the substrate. Taking into consideration the finding that cupric ions in the enzyme undergo reduction and oxidation (Friedman and Kaufman, 1965b; Blumberg et al., 1965), the equations were thus reformulated (Friedman and Kaufman, 1965b) :

$$E\begin{matrix}Cu^{++}\\Cu^{++}\end{matrix} + \text{ascorbate} \longrightarrow E\begin{matrix}Cu^{+}\\Cu^{+}\end{matrix} + \text{dehydroascorbate} + 2H^{+}$$

$$E\begin{matrix}Cu^{+}\\Cu^{+}\end{matrix} + O_2 + RH + 2H^{+} \longrightarrow E\begin{matrix}Cu^{++}\\Cu^{++}\end{matrix} + ROH + H_2O .$$

Subsequent kinetic studies by Goldstein et al. (1968) support the scheme of the catalytic reaction, in which the first product, dehydroascorbate, leaves the enzyme before the addition of the subsequent substrates (so-called ping-pong mechanism); the subsequent substrates (oxygen and dopamine) then add to the reduced enzyme intermediate before either product is released. Thus it seems obvious that ascorbate is important in its action as a reducing agent and electron donor in the catalytic reaction, and its function is presumably in the reduction of cupric ions in the enzyme molecule to cuprous form, which is catalytically active. Yet, in order to arrive at a complete understanding of the mechanism of this hydroxylation reaction, a closer examination of certain aspects of the role of ascorbate appears mandatory. Incidentally some analogous systems

which involve metal-containing enzymes have come to my attention. Tryptophan-2,3-dioxygenase is another copper-containing enzyme which requires enzyme-bound cuprous ion for its catalytic activity (Brady et al., 1971; Hirata and Hayaishi, 1971; Brady et al., 1972). Oxidative inactivation of this enzyme resulted in the formation of enzyme-bound cupric ions, and reductive activation resulted in the formation of cuprous ions (Cu^+), which was shown to be caused by a free radical, superoxide anion (O_2^-). This finding leads us to speculate that superoxide anion may also be the immediate mediator for the formation of cuprous ions in DBH. Since it has been shown that ascorbate acts as the cofactor in this regard, it was therefore proposed that the role of ascorbate is in the generation of superoxide anion, which subsequently reduces the cupric ion to cuprous form. Similar observations on the reductive reactivation by O_2^- of certain other metal-requiring enzyme, such as protocollagen proline hydroxylase, have been reported (Bhatnagar and Liu, 1972). Consequently, I planned to investigate the presence of superoxide anion in the ascorbate-dependent DBH system, and hoped to substantiate the role of ascorbate as the superoxide generator in this system.

When the DBH reaction is run in vitro, some other cofactors are also required. Fumarate is one of them, which is probably to facilitate the interaction of the reduced enzyme intermediate with oxygen and most likely induces a conformation change of the enzyme (Kaufman and Friedman, 1965; Goldstein et al., 1968). Fumarate may also speed the reoxidation of the cupric ion (Axelrod, 1972). The requirement for fumarate is not specific; other dicarboxylic acids such as succinate, α-ketoglutarate and malate, though less effective, are also active (Levin et al., 1960). Catalase is necessary to destroy peroxides formed by the auto-oxidation of ascorbate

and dopamine (Axelrod, 1972). Interestingly, DBH lacks substrate specificity and can beta-hydroxylate a wide variety of phenylethylamine derivatives including tyramine, phenylethylamine and amphetamine (Levin and Kaufman, 1961; Creveling et al., 1962). In fact, it was found that tyramine not only is a substrate, but may be a better one than dopamine. It should be noted, however, that the pH optimum for tyramine is 5.5, whereas that for dopamine is 6.2 (Creveling et al., 1962). Another striking feature of the DBH reaction *in vitro* is that the enzyme exhibits a broad pH range. It prefers a mildly acidic medium, with a pH optimum of 5.5. Changes in pH to 4-5 or 8-11 inactivate DBH without altering its molecular weight (Foldes et al., 1973).

Soluble Form and Membrane-Bound Form of DBH in Granules. Due to easy availability of large quantities of adrenal tissue, most of the studies of DBH have been done with bovine adrenal medulla. We know that DBH is closely associated with chromaffin granules, and is about equally distributed between soluble lysate and insoluble membranous residue of chromaffin granules of bovine adrenal medulla (Duch et al., 1968; Belpaire and Laduron, 1968; Winkler et al., 1970). When the rabbit adrenal medulla was studied, it was found that between 22 % and 52 % of total granular DBH is in soluble form (Viveros et al., 1969a, 1969c). During the process of exocytosis, this soluble portion of DBH is discharged into the extracellular space along with catecholamines and other soluble contents of the granule (for review, see Kirshner and Kirshner , 1971; Smith and Winkler, 1972). The evidence for exocytosis is similar but less compelling for sympathetic nerves (Smith, 1971; Weinshilboum et al., 1971a; Smith and Winkler, 1972; De Potter, 1973; Fillenz and West, 1973; Geffen and Rush, 1973), and for central noradrenergic neurons we can only speculate

by analogy. As mentioned above, it has been assumed that the process of catecholamine release in the central nervous system is similar to that described for the adrenal medulla and peripheral sympathetic system (Axelrod and Weinshilboum, 1972). Although up to 50 % of the DBH in chromaffin granules can be readily solubilized by osmotic lysis, only 10-20 % of the enzyme in noradrenergic vesicles of the sympathetic nerve is soluble under these conditions (De Potter et al., 1970; Smith et al., 1970; Weinshilboum et al., 1971a; De Potter, 1973; Fillenz and West, 1973; Geffen and Rush, 1973). Earlier Hörtnagl et al. (1969) had shown that upon lysis of a fraction obtained by gradient centrifugation of a microsomal sediment from splenic nerve, only 8 % of total DBH was recovered in the supernatant. Thus after the release of neurotransmitters in sympathetic nerve, a large portion of DBH remains within the neuronal structure. For the central nervous system, biochemical evidence concerning the nature of noradrenergic vesicles is meager. In a short communication, Belmar et al. (1974) analyzed the subcellular distribution of norepinephrine and DBH in the hypothalamus of rat. They demonstrated that after hypo-osmotic shock of a synaptosomal preparation, DBH activity in the soluble fraction is low (less than 10 %). This finding strongly suggests that the DBH in the hypothalamic noradrenergic nerve terminals is mainly present in the membrane-bound form, with only a small proportion in the soluble form. This may imply that, like peripheral sympathetic nerve, a large portion of DBH still remains within the neuronal structure after the process of exocytosis. Thus the DBH activity measured in homogenates of central nervous tissue can be a reasonable measure of the neuronal content of this enzyme; and only relatively small amounts will be in the extracellular space.

Fate of Released DBH. After DBH molecules are discharged from chromaffin cells or peripheral sympathetic terminals into the interstitial space, they enter the general circulation by way of venous channel and lymphatic drainage. There is no evidence showing the reuptake of DBH into neuronal structures. DBH activity has been detected in the serum (Weinshilboum and Axelrod, 1971; Rush and Geffen, 1972) and in the lymph collected from human thoracic duct during open chest surgery. There is no study concerning the passage of DBH after it is discharged from central noradrenergic terminals. By analogy, again, we speculate that it enters the extracellular space and subsequently gain access to the general circulation via venous drainage or ventricular system. There still remains unanswered the question of how it crosses the brain-blood barrier.

Transport of DBH in the Axon. Neurotransmitters can be synthesized in the nerve terminals as long as there are sufficient amounts of the enzymes required for biosynthesis. However, the enzyme proteins must be synthesized in the perikaryon and transported along the axon to the terminals. When peripheral noradrenergic nerve axons are ligated, there is a rapid accumulation of dense core vesicles proximal to the constriction, indicating a somatofugal transport of amine storage vesicles. Both biochemical (Laduron and Belpaire, 1968) and immunological (Geffen and Ostberg, 1969) studies indicate that DBH accumulates at a rapid rate proximal to a constriction in peripheral noradrenergic nerves. The rate of transport of amine storage granules in dog splenic nerves was calculated to be about 3 mm/hour (Laduron and Belpaire, 1968). The transport rate of DBH activity was estimated to be 1.61 mm/hour in intact rat sciatic nerve after ligation (Coyle and Wooten, 1972). In biopsy samples of normal human sural nerves incubated in vitro, the rate of accumulation of

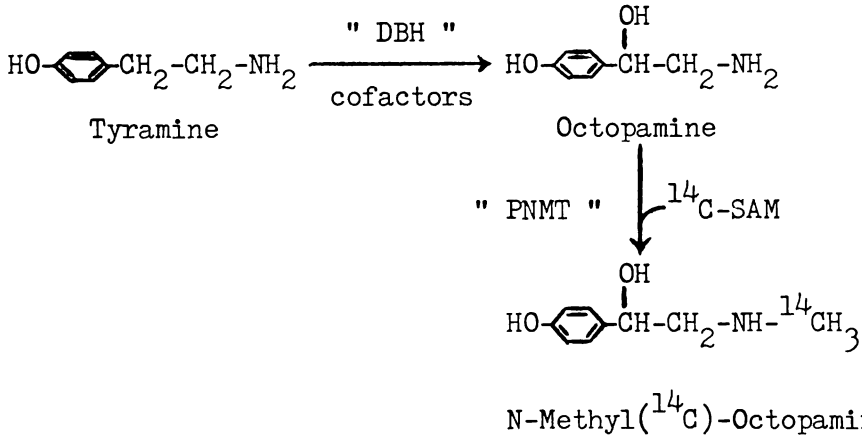
DBH above the ligature indicated that this enzyme was transported distally at a velocity of 2 mm/hour (Brimijoin et al., 1973). The rate of transport of DBH in central noradrenergic neurons is expected to be as rapid as it is in the peripheral nerves.

Endogenous Inhibitors of DBH. When assayed by a simple procedure without the addition of exogenous cupric ions, homogenates of adrenal medulla or sympathetically innervated structures show little or no DBH activity. However, activity is readily demonstratable after the homogenate has been fractionated (Levin et al., 1960; Austin et al., 1967). The marked increase in the units of enzyme activity which occurred during purification of the enzyme raised the possibility that endogenous inhibitors may be present (Creveling et al., 1962). DBH has been purified and has been shown to be a copper-containing protein. It appears that endogenous inhibitors of DBH, which are present in many tissues, act by binding to or interacting with the copper at the active site of the enzyme. DBH, purified from the bovine adrenal gland, is inhibited by sulfhydryl compounds such as cysteine, glutathione and mercaptoethanol (Nagatsu et al., 1967). The effect of the endogenous inhibitors is reversed by Cu^{++}, Hg^{++}, p-hydroxymercuribenzoate and N-ethylmaleimide (Duch et al., 1968). When the cupric ions are used to inactivate the endogenous inhibitors, optimal concentrations of Cu^{++} must be established for each tissue; as the optimal concentration of cupric ion is exceeded, the DBH activity is reduced (Molinoff et al., 1971). Although DBH is a copper-containing enzyme, in the absence of contamination by endogenous inhibitors it will be inactivated by even small amounts of free cupric ions (Duch et al., 1968). Hence in the tissue assay the exogenous cupric ion must be added in the amount just sufficient to neutralize the inhibitors present in the homoge-

nates. A very striking property of the DBH inhibitors is the enormous amount of activity which is found in various organs (Molinoff and Orcutt, 1973). The high concentration of inhibitory activity in many organs means that if even a small percentage of the inhibitors has access to DBH, they may be playing a significant physiological role. Although a large part of the inhibitory activity is in the soluble fraction, a certain percentage of it is found in association with the noradrenergic vesicles. Consistent with this observation is the fact that DBH-inhibitory activity has been found in washed adrenal chromaffin granules (Duch et al., 1968).

Measurement of DBH Content by In Vitro Methods. Until recently it was difficult to measure DBH activity in tissue because the existing methods lacked sensitivity and endogenous inhibitors interfered with the precise measurement of the enzyme. A sensitive and convenient procedure has been developed that made it possible to measure DBH in the homogenates of a wide variety of sympathetic structures of rats, including adrenal gland, sympathetic ganglia, heart and salivary gland (Molinoff et al., 1971). The assay involves coupled enzymatic reactions in which the β-hydroxylated product of the DBH reaction is made radioactive by the transfer of a ^{14}C-methyl group from S-adenosylmethionine [methyl-^{14}C]. The latter reaction is catalyzed by phenylethanolamine-N-methyltransferase (PNMT), an enzyme with a high degree of specificity for β-hydroxylated amines (Axelrod, 1962). The final reaction product is separated from the radioactive S-adenosylmethionine (^{14}C-SAM) by organic solvent extraction, and its radioactivity is determined. The assay uses either tyramine or phenylethylamine as the substrate, and endogenous inhibitors of DBH are inactivated by the addition of optimal concentrations of cupric ions. Many cofactors are also required for the reaction; it measures, therefore, the DBH activity in an optimized

condition, not representitive of the real <u>in vivo</u> situation. The reaction sequence, utilizing tyramine as the substrate, is :



This sensitive assay has been widely used to study the DBH activity in regions of rat brain during development (Coyle and Axelrod, 1972), after drug treatment (Reis and Molinoff, 1972), and after manipulation of different endocrine systems (Kizer et al., 1974). DBH activity in the brains of schizophrenic patients has also been measured (Wise and Stein, 1973; Wyatt et al., 1974). Hence I adopted this radioenzymatic assay to study the changes in DBH activity in regions of rat brain after manipulating the adrenocortical system in the rat.

Another recent advance in the study of DBH is the production of an antibody against highly purified adrenal DBH in immunized animals (Geffen et al., 1969; Hartman and Udenfriend, 1970). This antibody has been used for the immunocytochemical localization of DBH in neuronal structures (Fuxe et al., 1970b; Hartman et al., 1972), and for the radioimmunoassay of DBH in circulating blood (Rush and Geffen, 1972). The distribution of the hydroxylase in rat brain correlates well with the distribution of norepinephrine measured by the fluorometric method and by the Falck-Hillarp technique; thus it has been firmly established that dopamine-β-hydroxylase is a marker for central noradrenergic neurons.

==== C H A P T E R I - 7 ====

DOPAMINE-β-HYDROXYLASE AS ANOTHER REGULATORY ENZYME FOR THE BIOSYNTHESIS OF NOREPINEPHRINE

A complete understanding of how a metabolic pathway is regulated in the cell requires a knowledge of the rate-limiting step in that pathway. Usually it is at this "pacemaker" step that controls are exerted. In the study of hormonal effects on the enzymes for norepinephrine synthesis in the brain, knowledge of the identity of the rate-limiting step in the tyrosine-to-norepinephrine pathway is essential.

Tyrosine hydroxylase (TH), the first enzyme in the pathway, has been shown to be rate-limiting for the biosynthesis of catecholamines (Levitt et al., 1965). It is likely that the maximum rate of catecholamine synthesis is limited by the amount of tyrosine hydroxylase in the neuron. It is also true that stimulation of sympathetic nerves leads to increase in the rate of synthesis of catecholamines via an increase in the activity of tyrosine hydroxylase. Unfortunately this enzyme is present in both dopaminergic and noradrenergic neurons in the brain. Since I am interested primarily in changes of central noradrenergic neurons after endocrinological manipulation, I therefore studied DBH which is the marker enzyme for the noradrenergic neuron.

Dopamine-β-hydroxylase has been postulated to be another regulatory enzyme for the biosynthesis of norepinephrine (Molinoff and Orcutt, 1973). It is oversimplified to suggest that tyrosine hydroxylase is the only regulated step in this biosynthetic pathway. In fact, the identification of the pacemaker steps in any interlocking sequence of enzyme-catalyzed

reactions has proved to be a difficult task. Even in such well-studied metabolic pathways as glycolysis and respiration, the rate-limiting step is still a subject for speculation. In most cases, including that of norepinephrine biosynthesis, more information is needed on rates of degradation of intermediates, precursor and intermediate pool sizes, and compartmentalization of enzymes and substrates. Furthermore, it should be emphasized that the rate-limiting step for the same process may vary from one tissue to another, and obviously, the step need not be an enzymatic reaction per se. Thus, glucose metabolism in muscle is limited by the rate of its entry into the cell, whereas in liver, it is some other step that is rate-limiting. Moreover, even for the same tissue, the rate-limiting step for a metabolic pathway might not be the same in all species. For these reasons, it is an unwarranted oversimplification to speak of the rate-limiting step in norepinephrine biosynthesis, as if all tissues constituted a homogeneous continuum; the topic can be discussed only with reference to a specific organ in a specific animal (Kaufman and Friedman, 1965).

With more studies and more understanding on the nature of dopamine-β-hydroxylase, a clearer picture of how the conversion of dopamine to norepinephrine is regulated has emerged. In the first place, the enzymes and substrates of this pathway do not have free access to one another since dopamine-β-hydroxylase is sequestered in chromaffin granules and noradrenergic vesicles. Thus, the amount of dopamine entering the vesicles may determine the amount of norepinephrine formed. Very little is known at the present time concerning possible restrictions to the transport of dopamine into these vesicles. It has been shown that inhibition of the uptake of dopamine into vesicles by reserpine is associated with a marked

decrease in the conversion of dopamine to norepinephrine (Rutledge and Weiner, 1967). In a study of the biosynthesis of cardiac norepinephrine using radioactive tyrosine and dopamine as precursors, Landsberg et al. (1969) suggested that dopamine access to the vesicle may be rate-limiting under certain physiological conditions. In the second place, the catecholamine biosynthetic pathway is highly branched. Some of the dopamine is taken up into vesicles and converted to norepinephrine, and some is deaminated by the intraneuronal monoamine oxidase. A competition therefore exists between deamination and uptake/beta-hydroxylation. Any situation which increases the capacity of the neuron to catabolize dopamine would reduce the formation of norepinephrine. Thirdly, the availability of the essential cofactors (ascorbate and oxygen) may also affect the reaction rate. For instance, hypoxia has been shown to alter the synthesis of norepinephrine and, to the contrary, the administration of large amount of ascorbic acid increased the norepinephrine content of the rat brain (Izquierdo et al., 1968). Moreover, in the presence of endogenous inhibitors dopamine-β-hydroxylase can become the rate-limiting enzyme for the norepinephrine synthesis (Molinoff and Orcutt, 1973). If the DBH inhibitors are playing a physiological role in regulating DBH activity, then it should be possible to demonstrate that their cellular and subcellular location is such that they have access to the enzyme. Normally there is so much inhibitory activity present that even if only a small percentage of it is in the noradrenergic nerve terminal, it will play a significant role by limiting the activity of DBH and thus modulate norepinephrine biosynthesis. Duch et al. (1968) have shown that DBH-inhibitory activity could be found in washed adrenal chromaffin granules. Recent experiments have also suggested that DBH inhibitors do indeed have access to DBH.

When homogenates of rat heart are subjected to sucrose density gradient centrifugation, a significant amount of inhibitory activity is found in the same region of the gradient as are noradrenergic storage vesicles (Molinoff and Orcutt, 1973).

One of the arguments that has been cited in favor of tyrosine hydroxylation being the rate-limiting step in norepinephrine synthesis is that the concentration of tyrosine in tissue is about 10^{-4}M, whereas dopa and dopamine are not normally detectable (Nagatsu et al., 1964). This argument is strong, since in a linear sequence of reactions only the substrate for the rate-limiting step would be expected to accumulate to any extent. However, the data serve not only to strengthen the case for tyrosine hydroxylation being the limiting step in the adrenal tissues of most animals, but they also weaken it for other animals or other tissues. In most animals studied, it has been found that the amount of dopamine is either very low or not detectable (Shepherd and West, 1953). In the adrenal gland of the sheep, however, the concentration of dopamine is quite high (Shepherd and West, 1953). Furthermore, it is known that in the brain there are high levels of dopamine and dopa is also detectable (Carlsson, 1959). Sympathetic nerve cells contain dopamine as well as norepinephrine (Schümann, 1958). In the brain, dopamine is the neurotransmitter for dopaminergic neurons; and it is also accumulated in noradrenergic neurons as the precursor for norepinephrine synthesis. Many studies have indicated that the decrease of norepinephrine in the central neurons is faster after inhibition of dopamine-β-hydroxylase with disulfiram (Goldstein and Nakajima, 1967), α,α'-dipyridyl (Bapna et al., 1970) or FLA 63 (Persson and Waldeck, 1970) than after inhibition of the first step of norepinephrine synthesis with α-methyl-p-tyrosine (α-MT). One obvious possibility to

account for this phenomenon is the availability of stored dopamine for norepinephrine synthesis after tyrosine hydroxylase inhibition (Goldstein and Nakajima, 1967). In another study, Javoy and Glowinski (1971) failed to observe an immediate decrease of norepinephrine in the cortex of the rat after α-MT although dopamine levels in dopaminergic ternimals of the striatum were diminished very rapidly. This finding also supported the hypothesis of the availability of stored dopamine for norepinephrine synthesis. In a more recent report Thierry et al.(1971a) confirmed that there is a large amount of dopamine stored in the noradrenergic terminals of the rat cerebral cortex. This finding supported the notion that dopamine-β-hydroxylase may occupy another gate that limits the conversion of precursor to norepinephrine. Most of the dopamine stored in noradrenergic terminals appears to be available for norepinephrine formation, and this stored dopamine represents a reservoir which can be used for norepinephrine synthesis when the first step of norepinephrine biosynthesis is blocked. Thus, the last as well as the first step of norepinephrine biosynthesis could be important in regulating norepinephrine formation in the brain. Both the transformation of tyrosine to dopa and the conversion of dopamine to norepinephrine may be rate limiting (Bartholini and Pletscher, 1968; Thierry et al., 1971a; Kopin, 1972, p.139; Stolk, 1973). The failure to detect an appreciable increase in brain norepinephrine levels, although dopamine content rose markedly after peripheral administration of large dose of dopa (Carlsson, 1959, 1964; Everett and Borcherding, 1970; Weiss et al., 1972) is another and complementary argument in favor of a rate limiting role of the last step in norepinephrine biosynthesis.

Additional support for the conclusion that, DBH, as well as tyrosine hydroxylase, is rate-limiting and can in turn be subject to regulation

comes from the fact that a number of procedures produce similar changes in the levels of tyrosine hydroxylase and dopamine-β-hydroxylase, but not that of dopa decarboxylase (Thoenen et al., 1971) or PNMT (Viveros et al., 1969b). These procedures include (1) the administration of drugs like reserpine which lead to an increase in TH and DBH in the adrenal, sympathetic ganglia and in sympathetically innervated organs (Mueller et al., 1969; Molinoff et al., 1970); (2) the administration of 6-hydroxydopamine which leads to similar increases of TH and DBH in the adrenal medulla (Brimijoin and Molinoff, 1971); (3) the administration of nerve growth factor which leads to marked increases in TH and DBH in the superior cervical ganglion of the rat (Thoenen, 1972a); and (4) various types of stress including immobilization (Kvetnansky et al., 1970, 1971) and cold-stress (Thoenen et al., 1971) which induce apparently specific changes in DBH and TH. That the neurally mediated control of these two enzymes is not regulated as a single operational unit is illustrated by the fact that the turnover of DBH in the superior cervical ganglion is more rapid than that of TH (Thoenen et al., 1971). Also, in studying the effect of reserpine on the adrenal glands, Patrick and Kirshner (1971b) found that the changes in the activity of TH and DBH are not linked.

==== C H A P T E R I - 8 ====

NEURAL AND HORMONAL REGULATION OF DOPAMINE-β-HYDROXYLASE

Chemical transmitters in sympathetic nerves are in a state of flux, continually being synthesized, released, metabolized and recaptured. In spite of all these dynamic changes the amount of catecholamines in tissues remains rather constant. This is because of a variety of adaptive mechanisms that alter the formation and disposition of catecholamines. Some of them involve the regulation of the catecholamine-synthesizing enzymes by the neural as well as the hormonal control mechanisms (Axelrod, 1971; Axelrod, 1972; Axelrod and Weinshilboum, 1972; Thoenen, 1974). Most of the studies were done with adrenal medulla and peripheral sympathetic structures, which revealed that the neural effect is responsible for short- and ling-term regulation of those enzymes, whereas the hormonal induction is predominantly a long-term regulatory process.

Neural Regulation of Enzymes. When sympathetic nerves are stimulated, the conversion of tyrosine to norepinephrine in them is rapidly increased (Weiner and Rabadjija, 1968). The increased nervous activity specifically affects tyrosine hydroxylase because its activity is affected by dopamine and norepinephrine by the mechanism of end-product inhibition (Spector et al., 1967; Weiner and Rabadjija, 1968). Any increase in nerve-firing brought on by stress, cold and certain drugs lowers the level of catecholamines in the nerve terminals. This reduces the negative-feedback effect of dopamine and norepinephrine on tyrosine hydroxylase, so that more tyrosine is converted to dopa, which in turn is converted to make more

catecholamines. It has been suggested that this rapid adaptation only involves a change in enzyme activity without an increase in the total amount of enzyme molecules (Sedvall and Kopin, 1967; Dairman et al., 1968).

A slower regulatory process is brought on by prolonged firing of sympathetic nerves, which can step up the manufacture of the catecholamine-synthesizing enzymes tyrosine hydroxylase, dopamine-β-hydroxylase and, to a lesser extent, PNMT; the rise in the enzyme level enables the nerves to make more neurotransmitter. It has been shown that tyrosine hydroxylase is induced by a transsynaptic neural mechanism in response to prolonged stimulation (Thoenen, 1974). Nonetheless, unstimulated peripheral sympathetic neurons and adrenal medullary cells seem not to be dependent upon intact neural inputs for the maintenance of their tyrosine hydroxylase activity (Thoenen, 1970; Patrick and Kirshner, 1971a) : Denervation or decentralization may reduce the dynamic turnover of tyrosine hydroxylase, but not its static tissue content. In the central nervous system, a significant increase in tyrosine hydroxylase after a two-day exposure to cold was restricted to the medulla oblongata; there was no rise in the hypothalamus or the rest of the brain (Thoenen, 1970). In addition, Hulme et al. (1974) failed to obtain any increase in tyrosine hydroxylase activity in homogenate of rat brain after 3-day or 7-day treatment of reserpine, which is in contrast to the results obtained by others (Mueller et al., 1969; Segal et al., 1971; Besson et al., 1973; Zigmond et al., 1974). Various experimental results indicate that tyrosine hydroxylase in the brain appears to be relatively insensitive to stimuli which lead to marked changes in its levels in the peripheral sympathetic neurons. This may reflect differences between peripheral and central neurons in terms of some morphological and functional features.

Similar observations on the transsynaptic induction of dopamine-β-

hydroxylase in peripheral sympathetic neurons were obtained in response to prolonged stimulation (Molinoff et al., 1970, 1972; Molinoff and Orcutt, 1973). The administration of reserpine for 6 days caused an increase in DBH in rat adrenals, sympathetic ganglia and heart (Molinoff et al., 1970). The effect of reserpine on DBH was dose-dependent, was reduced by ganglionic blocking agent, chlorisondamine, and was prevented by the inhibitor of protein synthesis, cycloheximide (Molinoff et al., 1972). When the preganglionic nerve to the superior cervical ganglion was cut unilaterally and reserpine was administered, there was an elevation in DBH in the innervated but not in the decentralized ganglion (Molinoff et al., 1970). Another interesting finding in this study was that in the absence of reserpine, the DBH activity in the ganglia of decentralized side was no lower than that of the control side after 6 days. This again suggests that when not stimulated, the peripheral sympathetic neurons are not dependent on intact neural inputs to maintain their DBH content. The situation in the adrenal gland is somewhat controversial : while Thoenen et al. (1971) and Patrick and Kirshner (1971a) found no change in DBH activity in the denervated adrenal compared to that of intact side, Kvetnansky et al. (1971) demonstrated that DBH activity was lower in the denervated adrenal gland than in the intact gland. At any rate, it appears that adrenal chromaffin cells are not absolutely dependent on intact neural inputs for the maintenance for their DBH activity. In the adrenals of growing young rats, however, the gradual increases in the content of tyrosine hydroxylase and dopamine-β-hydroxylase are dependent on the continuous trophic influences of preganglionic nerves (Patrick and Kirshner , 1972). In order to determine whether reserpine could increase DBH activity in the brain, the drug was administered (3 mg/kg, subcutaneously) for 3 days and the rats were killed on the fourth day to have DBH activity measured in the brain

and adrenals (Reis and Molinoff, 1972). While increasing DBH activity two-fold in the adrenal gland, reserpine did not change the activity of the enzyme in the brain. This finding is similar to that for the effect of reserpine on tyrosine hydroxylase in the brain (Hulme et al., 1974).

Thus it is clear that in response to a wide variety of prolonged stimulation, tyrosine hydroxylase and dopamine-β-hydroxylase can be induced in adrenal medulla and peripheral sympathetic neurons by a transsynaptic mechanism leading to the synthesis of new protein, whereas under resting conditions, the maintenance of the enzyme content does not depend on intact neural inputs. In the central nervous system both TH and DBH are less sensitive to stimuli which usually lead to marked changes in levels of these enzymes in the adrenal gland and peripheral sympathetic neurons.

Hormonal Regulation of Enzymes. With respect to the hormonal control of DBH and other enzymes for the metabolism of catecholamines, only a few studies have been reported. Hypophysectomy has been shown to reduce the activity of PNMT (Wurtman and Axelrod, 1965, 1966) and TH (Mueller et al., 1970) in the adrenal. ACTH can restore both PNMT and TH in hypophysectomized rat adrenals. Dexamethasone in very large doses increases adrenal PNMT (Wurtman and Axelrod, 1966) but not TH (Mueller et al., 1970) in hypophysectomized rats. Weinshilboum and Axelrod (1970) showed that the concentration (per unit weight), but not the total activity (per organ) of cardiac dopamine-β-hydroxylase, increases 3 weeks after hypophysectomy and is unchanged after treatment with either ACTH or dexamethasone for 5 days. On the other hand, rat adrenal DBH activity decreases 3 weeks after removal of the pituitary and is elevated after treatment with ACTH for 5 or 10 days, but is unchanges after daily dexamethasone treatment for 5 days. The activity after treatment with ACTH, however, is only restored to half

of the activity found in the adrenals of sham-operated animals. Furthermore, daily administration of ACTH (4 units, subcutaneously) for 5 days does not increase adrenal DBH in rats with intact pituitary glands. Gewirtz et al. (1971) confirmed the finding that hypophysectomy decreased DBH activity in the adrenal medulla after 7 days, and this decrease is still present after 180 days. Treatment with ACTH or dexamethasone for 6 days restores adrenal levels of DBH, but thyroxine is ineffective in this instance. Axelrod and Weinshilboum (1972) have presented a scheme to describe the regulation of biosynthetic enzymes in the adrenal medulla. In that diagram they proposed that the stress-induced increase in tyrosine hydroxylase activity is due mainly to the neural activity, that of DBH is due to both nerve activity and ACTH-corticosteroids, and PNMT activity is elevated mainly by adrenocortical hormones.

The effect of hormones on the catecholamine-synthesizing enzymes in peripheral sympathetic neurons has not been adequately studied. Using isolated rat superior cervical ganglia, Keen and McLean (1974) have demonstrated that dexamethasone (10^{-4} M) or dibutyryl-cyclic AMP (10^{-3} M) increased DBH content in those ganglia after 6 hours of incubation. In rats adrenalectomized for one week, the resting serum DBH activity was not different from that of control rats, and the elevations of serum DBH after immobilization stress were of same magnitude in both groups of rats (Weinshilboum et al., 1971b). Since most of circulating DBH molecules are released from sympathetic nerve terminals after removal of the adrenal glands, this implies that the manufacture and release of DBH from sympathetic neurons is not dependent on adrenocortical hormones. Conversely, rat serum DBH activity rose continuously after hypophysectomy and doubled within 5 weeks (Lamprecht and Wooten, 1973). This increase was not reversed by replacement doses of ACTH. On the other hand, vasopressin

reduced the increased levels of serum DBH activity in hypophysectomized rats to those found in sham-operated control animals, but had no effect on serum enzyme activity in sham-operated controls. It appears that the reduced intravascular volume and subsequent sympathetic activation accounts for the elevation of serum DBH levels, rather than the direct effect of hormones on the neurons.

The effects of pituitary-adrenocortical hormones on the catecholamine-catabolizing enzymes have also been studied. Monoamine oxidase in the sympathetically innervated organs was increased after hypophysectomy (Landsberg et al., 1969) and adrenalectomy (Westfall and Osada, 1969; Csaba et al., 1972; Parvez and Parvez, 1973), whereas glucocorticoid treatment decreased it (Parvez and Parvez, 1973; Petrović and Janić, 1974). COMT activity also increased after adrenocortical insufficiency and decreased by glucocorticoid treatment (Parvez and Parvez, 1973).

Some reports concerning the effect of endocrinological manipulations on TH and DBH activities in brain regions have been published. Since catecholamines have been implicated in the regulation of anterior pituitary secretion, the study of changes in the enzyme activity in various endocrine states may help to elucidate the site and the mechanism of feedback action of target hormones. Earlier Lipton et al. (1968) showed that there was a marked acceleration of synthesis of ^{14}C-norepinephrine from ^{14}C-tyrosine in the heart, spleen and adrenal of the hypothyroid rat, with only small changes in the brain. When brain homogenate was analyzed for tyrosine hydroxylase activity, no parallel change could be detected. Beattie et al. (1972) demonstrated that TH in rat hypothalamus was elevated 4 days after ovariectomy and rose progressively through day 60. Daily injection of estrogen for 1 week to ovariectomized rats caused a further increase

in TH, whereas progesterone was able to reduce the elevated TH levels. Thus they concluded that catecholamine synthesis in the rat hypothalamus may be altered by ovarian steroids, with estrogen having a stimulatory effect and progesterone an inhibitory effect. Using a recently developed microdissection method, Kizer et al. (1974) removed individual hypothalamic nuclei from the brains of adult male rats, and measured TH and DBH activities in pools of each nucleus 9 days after gonadectomy, thyroidectomy or adrenalectomy. Following orchidectomy there was a significant rise in the TH activity in the median eminence; this increased TH activity was partially reversed by treatment with testosterone. Thyroidectomy also induced an increase in the TH activity in the median eminence as well as some other hypothalamic nuclei. Again the treatment with thyroxine was able to reduce the elevated TH activity in those nuclei. On the contrary, adrenalectomy produced a fall in TH activity in the median eminence, which could be restored by dexamethasone treatment. Strikingly, DBH activity in hypothalamic nuclei was not altered by any of those endocrine manipulations, nor by treatment with reserpine for 7 days. It was proposed that hypothalamic catecholaminergic nerve terminals may participate in the feedback regulation of the gonads, thyroid and adrenals, and that these terminals are located in the median eminence. Although they were able to demonstrate the site of hormone effect in a discrete hypothalamic region, they could not determine whether the observed changes in TH activity reflected alterations in the activity of dopaminergic neurons, noradrenergic neurons, or both. Moreover, measurement of <u>in vitro</u> enzymatic activity in an artificial assay system may be misleading because it does not represent the real enzymatic activity in living organisms. Therefore, I measured the DBH activity, catecholamine content and norepinephrine forma-

tion in regions of rat brain after changes in adrenocortical state. In this way the effect of adrenocortical hormones on the functional state of central noradrenergic neurons can be more adequately assessed.

==== C H A P T E R I - 9 ====

EFFECT OF STRESS ON CENTRAL NORADRENERGIC NEURONS

Many stressful stimuli that increase ACTH secretion are also associated with changes in the functional activity of central noradrenergic neurons (Yuwiler, 1971; Van Loon, 1973). Hypoglycemia (Vogt, 1954) and hemorrhage (Coleman and Glaviano, 1963) decreased brain norepinephrine content. After five minutes of scuffling, there was a significant decrement in the content of norepinephrine in various segments of the brains of submissive mice (Bliss and Zwanziger, 1966). Another psychic stress in the form of witnessing a fight also decreased norepinephrine content in the brain stem of mice (Welch and Welch, 1968a). A comprehensive survey of the effect of more frequently used stressful procedures, such as electric shock, cold exposure, immobilization and muscular exercise, on the norepinephrine content and turnover in the brain is presented in Tables I-2~I-5 in the following pages. In essence, stress appears to decrease steady-state levels of brain norepinephrine in acute stage, and increase the dynamic turnover of it in both acute and chronic phases. It seems that the increase in the brain norepinephrine metabolism is primarily due to increased nervous impulse flow, rather than a change secondary to the increased adrenocortical secretion.

Cold stress has been reported to change tyrosine hydroxylase activity. While Gibson et al. (1969) found no change in TH activity in the homogenate of the whole brain of the rat after one to twenty-one days of cold exposure, Thoenen (1970) demonstrated an increase in TH activity in the medulla oblongata of the rat exposed to cold for one to four days; there was no

significant rise of TH activity in the hypothalamus or the residual parts of the brain. In comparison with the periphery, the increase in TH activity was considerably smaller in the medulla oblongata. Zigmond et al. (1974) were able to measure TH activity in the homogenate of the locus coeruleus and the rest of the brain stem, respectively. They found that although the locus coeruleus sample represented only about 3 % of the weight of the entire pons and medulla, it contained 23 % of the tyrosine hydroxylase activity present in that part of the brain. After 66 hours at 4°C, there was an 84 % increase in TH activity in the locus coeruleus of the rat. Repeated immobilization for 4 weeks induced an increase in TH activity in the hypothalamus of the rat, with no significant change in catecholamine content (Lamprecht et al., 1972). Four weeks after immobilization had been terminated, the tyrosine hydroxylase activity in the hypothalamus still remained elevated.

Dopamine-β-hydroxylase activity in the brain after stress has not been studied. I measured DBH activity in regions of rat brain after various stressful procedures, such as injection, laparotomy and immobilization. The effect of anesthetic agents such as ether (also a stressor for the rat) and pentobarbital was also studied.

TABLE I-2 Effect of Electric Shock on Norepinephrine in the Brain*.

Year	Authors	NOREPINEPHRINE Content	Turnover	Brain Regions	Animals
1963	Paulsen & Hess	Dec.		Whole brain	Guinea pig
1964	Maynert & Levi	Dec.		Brain stem	Rat
1964	Moore & Lariviere	Dec.		Whole brain	Rat
1966	Bliss & Zwanziger	Dec.		Various regions	Guinea pig
1966	Ordy et al.	Dec.		Hypothalamus	Monkey
1967	Kety et al.	Inc. (after repeated convulsive shocks)	Inc.	Various regions	Rat
1968	Bliss et al.	Dec.	Inc.	Whole brain & various regions	Rat
1968	Thierry et al.	N.C. (mild foot shock)	Inc.	Brain stem-midbrain	Rat
1969	Taylor & Laverty (1969b)	Dec.	Inc.	Various regions	Rat
1971	Thierry et al. (1971b)		Inc.	Brain stem	Rat
1973	Korf et al.		Inc.	Cerebral cortex	Rat

* Dec. = Decreased; Inc. = Increased; N.C. = No Change.

TABLE I-3 Effect of Cold Stress on Norepinephrine in the Brain*.

Year	Authors	NOREPINEPHRINE Content	Turnover	Brain Regions	Animals
1961	Moore et al.	N.C. (long exposure)		Whole brain	Rat
1964	Maynert & Levi	Dec.		Brain stem	Rat
1966	Gordon et al.	N.C.	Inc.	Whole brain	Rat
1967	Cox & Potkonjak	N.C.		Whole brain	Rat
1967	LeBlanc et al.	N.C.		Hypothalamus	Mouse
1967	Ingenito & Bonnycastle	N.C. (short exposure)		Whole brain & hypothalamus	Rat
		Inc. (long exposure)		Whole brain	Rat
1967	Welch & Welch		Dec. (long exposure)	Cortical & subcortical	Mouse
1967	Corrodi et al.	N.C.	N.C.	Whole brain	Rat
1969	Gibson et al.	N.C.	N.C.	Whole brain	Rat
1973	El-Halawani et al.	N.C.	Inc.	Whole brain	Turkey

* Dec.= Decreased; Inc.= Increased; N.C. = No Change.

TABLE I-4 Effect of Immobilization on Norepinephrine in the Brain*.

Year	Authors	NOREPINEPHRINE Content	Turnover	Brain Regions	Animals
1964	Moore & Lariviere	N.C.		Whole brain	Rat
1966	Bliss & Zwanziger	Dec.		Various regions	Guinea pig
1968	Carr & Moore	N.C.		Whole brain	Rat
1968	Welch & Welch (1968b)	N.C.		Whole brain & various regions	Mouse
1968	Corrodi et al.	N.C.	Inc.	Whole brain, hypothalamus, spinal cord	Rat
1971	Corrodi et al.	N.C.	Inc.	Whole brain, hypothalamus, cerebral cortex	Rat
1972	Nagura	Dec.		Whole brain	Rat
1972	Lamprecht et al.	N.C.		Hypothalamus	Rat

TABLE I-5 Effect of Muscular Exercise on Norepinephrine in the Brain*.

Year	Authors	NOREPINEPHRINE Content	Turnover	Brain Regions	Animals
1962	Freedman et al.	Dec.		Whole brain	Rat
1963	Barchas & Freedman	Dec.		Whole brain	Rat
1964	Moore & Lariviere	Dec.		Whole brain	Rat
1966	Bliss & Zwanziger	N.C.		Whole brain	Rat
1966	Gordon et al.	N.C.	Inc.	Whole brain	Rat
1967	Matussek et al.	Dec.		Midbrain	Rat
1971	Stone	Dec.	Inc.	Hypothalamus	Rat

* Dec.= Decreased; Inc.= Increased; N.C. = No Change.

==== C H A P T E R I - 10 ====

EFFECT OF PITUITARY-ADRENOCORTICAL HORMONES ON THE METABOLISM OF NOREPINEPHRINE

Landsberg and Axelrod (1968) reported that cardiac norepinephrine turnover was markedly increased in rats one month after hypophysectomy. Thyroidectomy and adrenalectomy were also associated with increased cardiac norepinephrine turnover, whereas ovariectomy was not. Chronic treatment of hypophysectomized rats with thyroxine and ACTH restored the turnover to normal, the replacement of thyroxine being more effective. They concluded that thyroid deficiency and, to a lesser extent, adrenal deficiency are associated with the increase in cardiac norepinephrine turnover mediated by an increase in sympathetic nervous activity. Westfall and Osada (1969) and Dailey and Westfall (1970) showed that adrenalectomized rats had an increased turnover of norepinephrine in the heart and that this change could be prevented by deoxycorticosterone acetate but not by hydrocortisone or corticosterone. They suggested that DOCA prevented a reflexly mediated increase in sympathetic activity by maintaining a normal blood pressure, thereby reversing the increase in norepinephrine turnover.

The relationship between pituitary-adrenocortical hormones and the disposition of norepinephrine in the brain is less clear. Norepinephrine content in the brain did not change after adrenalectomy (Pfeifer et al., 1963) or hypophysectomy (Landsberg and Axelrod, 1968; Versteeg et al., 1972). Changes in central turnover of norepinephrine were studied by Javoy et al. (1968) at various time intervals after adrenalectomy in the rat. Norepinephrine turnover was not affected shortly after the operation,

but was accelerated six days later. In a single study Landsberg and Axelrod (1968) demonstrated that more than one month after hypophysectomy the turnover of norepinephrine in rat brain did not change, whereas Versteeg et al. (1972) found a decrease in norepinephrine turnover in rat brain 2-3 weeks after hypophysectomy. Fuxe and co-workers have carried out extensive investigations on the effect of manipulation on pituitary-adrenal system on central aminergic neurons by biochemical and histofluorescence analyses (Fuxe et al., 1973a, 1973b). The results with respect to the effect on the turnover of norepinephrine are summarized in Table I-6.

Table I-6 Changes in Norepinephrine Turnover in Rat Brain after Adrenalectomy (AdX), Hypophysectomy (HypX), and Hormonal Replacement. (From Fuxe et al., 1973a, 1973b).

*Changes are the comparison between the experimental animals and the sham-operated controls.

Operation	Hormone Injection	Circulating Corticoids*	Circulating A C T H*	Norepinephrine Turnover*
AdX	-	Decreased	Increased	Increased
HypX	-	Decreased	Decreased	Decreased
-	B, F, Dex	Increased	Decreased	No Change, or Slightly Increased
-	ACTH	Increased	Increased	Slightly Increased
AdX	B, F, Dex, DOC	No Change or Incr.	No Change or Incr.	No Change
HypX	Dex	No Change or Incr.	Decreased	Decreased
HypX	ACTH	No Change or Incr.	No Change or Incr.	Decreased

B = Corticosterone; F = Hydrocortisone; Dex = Dexamethasone; DOC = Deoxycorticosterone.

It appears difficult to draw any definite conclusion from their data. The increased norepinephrine turnover after adrenalectomy is consistent with the report by Javoy et al. (1968), whereas the result with respect to hypophysectomy is not compatible with that of others (Landsberg and Axelrod, 1968). The effects of exogenous hormone administration to the intact vs. hormone-deficient rats are also poorly correlated. It seems likely that in adrenalectomized rats the increase in norepinephrine turnover is partly mediated via the increase in ACTH secretion, and partly due to the loss of adrenal corticosteroids (Fuxe et al., 1973a). Exogenous corticosteroids, on the other hand, may have slightly inhibitory effect on norepinephrine turnover.

Glucocorticoids have been shown to decrease the uptake of norepinephrine into the heart (Iversen and Salt, 1970). Direct effects of glucocorticoids on noradrenergic nerve terminals have also been demonstrated. Maas and Mednieks (1971) found that slices of rat cerebral cortex, when preincubated with hydrocortisone, took up more norepinephrine from the media than did the control preparations. Similar findings have also been obtained in the rat by Fuxe et al. (1973a) with corticosterone. Keen and McLean (1974) showed that incubation with dexamethasone increased DBH content in isolated rat superior cervical ganglia but did not affect norepinephrine levels, whereas incubation with dibutyryl-cyclic AMP increased both.

In summary, there is a good deal of evidence indicating that hypophysectomy or adrenalectomy is associated with an increase in the turnover of norepinephrine in tissues other than brain. This increase in turnover can be reversed by appropraite hormonal replacement. On the contrary, reports dealing with the relationship between corticosteroids and brain norepine-

phrine are less congruent. In view of the important role played by central noradrenergic neurons in the control of pituitary-adrenocortical secretion, further studies to delineate the changes of this neuronal system under various adrenocortical states seem mandatory. By and large, the experimental work of my dissertation is designed to deal with this physiological problem.

P A R T II

E X P E R I M E N T A L

==== C H A P T E R II - 1 ====

PRELIMINARY DOPAMINE-β-HYDROXYLASE ASSAY

OBJECTIVE 1. To set up a preliminary DBH assay for the determination of DBH activity in fractions of Sephadex column eluate of beef adrenal medullary homogenate.

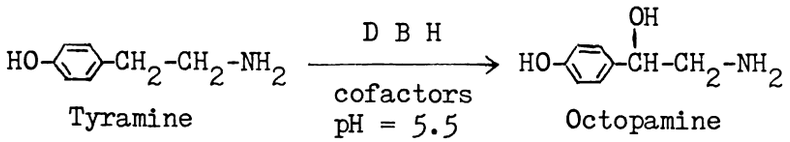
2. To set up PNMT assay for the determination of PNMT activity in fractions of Sephadex column eluate, and for the second step of coupled reaction for assaying DBH activity.

3. To isolate PNMT from beef adrenal medulla, which is required for the second step of coupled reaction for assaying DBH activity.

4. To isolate DBH from beef adrenal medulla, which will be used for subsequent studies on the biochemical properties of DBH.

INTRODUCTION The assays which have been used to measure DBH activity are time consuming and relatively insensitive for the measurement of DBH activity in tissue homogenates. In 1971, Molinoff et al. reported a radioenzymatic assay which is specific and is sufficiently sensitive to measure DBH activity in a variety of organs. The principle of the assay has been described in Chapter I-6 (pp. 34-35). The reaction sequence for this assay when tyramine is the substrate is shown as :

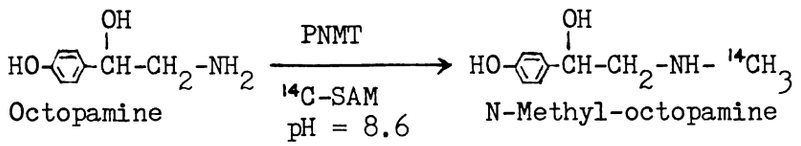
Step 1. DBH reaction



Tyramine Octopamine

(cofactors : cupric ions, ascorbate, fumarate, catalase)

Step 2. PNMT reaction



An essential part for the DBH assay is to prepare PNMT which is required for the second step of the coupled enzymatic reactions.

METHODS AND RESULTS

A. Isolation of PNMT and DBH from Beef Adrenal Glands.

The beef adrenal glands were obtained fresh from a local slaughter house through the courtesy of Dr. A. Roy. The purification procedure was a modification of that described by Molinoff et al. (1971) and was carried out in a cold room at 4-6°C. Medullary tissue was dissected out quickly from adrenal glands and was homogenized in 3 volumes of 1.15 % KCl in a commercial blender for one minute. Ordinarily 10 beef adrenal glands will give rise to about 30 grams of medullary tissue. The homogenate was centrifuged for 40 minutes at 100,000 x g. After aspiration of the fat which rose to the upper layer, the precipitate was discarded and the supernatant was collected. Solid ammonium sulfate was slowly added to the supernatant fluid to a final saturation of 30 % (17.6 gm/100 ml). After centrifugation at 10,000 x g for 10 minutes, the precipitate was discarded and additional ammonium sulfate was added to the supernatant fluid to a final saturation of 50 % (an additional 12.6 gm/100 ml). After centrifugation at 10,000 x g for 10 minutes, the supernatant fluid was discarded and the precipitate was then dissolved in 10-15 ml of 0.001 M Tris buffer, pH 7.4. The solution was dialyzed against 4 liters of 0.01 M Tris buffer, pH 7.4 for two times, 8 hours each. The dialyzed preparation was centri-

fuged at low speed to remove the flocculent substance and aliquots (about 20 ml) were passed over a Sephadex G-200 column (Pharmacia Fine Chemicals), 3 x 80 cm. The column was eluted with 0.05 M Tris buffer, pH 7.4 and 8-ml fractions were collected by an automatic fraction collector. Aliquots of column eluate from each tube were read in a spectrophotometer at 280 mμ and 260 mμ to determine the protein concentration. The elution curve with respect to protein concentration is shown in Fig. 1-1 (p. 65).

B. PNMT Assay.

Chemicals and Reagents. (1) DL-Octopamine HCl, borax (sodium tetraborate), PPO and POPOP were purchased from Sigma Chemical Company, St. Louis. (2) Disodium ethylenediaminetetraacetate was a product of Fisher Scientific Company, Fair Lawn, New Jersey. (3) S-Adenosyl-L-methionine-methyl-^{14}C (specific activity 50-60 mCi/mmole) was purchased from New England Nuclear Corporation, Boston. (4) Toluene (Scintill AR) and isopentyl alcohol were purchased from Mallinckrodt Chemical Works, St. Louis.

PNMT was assayed by a modification of a previously described procedure (Axelrod, 1962). Octopamine was used as the substrate. The reaction mixture consisted of 20 μl of Sephadex column eluate containing PNMT, 200 ng of octopamine HCl in 200 μl of 0.01 N HCl solution, and 100 μl of freshly pre-mixed solution containing 80 μl of 1 M Tris buffer, pH 8.6, 10 μl of EDTA solution (470 mg of disodium EDTA in 100 ml of water, pH 8.6) and 10 μl of ^{14}C-SAM (1.2 nmoles). The reaction was run in a total volume of 320 μl in a 15-ml test tube (16 x 100 mm) at 37°C for 30 minutes and was then stopped by the addition of 0.5 ml of 0.2 M sodium borate buffer, pH 10. The reaction product, ^{14}C-N-methyl-octopamine, was extracted into 6 ml of a mixture of toluene and isopentyl alcohol, 3:2 (v/v) by vigrous mixing for 15 seconds on a mixer (Supermixer, Lab-Line, Melrose Park, Illinois).

After centrifugation at low speed, 4 ml of the organic phase were transferred to counting vial containing an additional 2 ml of toluene-isopentyl alcohol mixture (3:2). The sample were then dried in an oven connected to the vacuum suction, at the temperature of 80-90°C. This drying step is necessary to remove volatile radioactive contaminants which are extracted into the toluene-isopentyl alcohol (Molinoff et al., 1969). After drying, which usually takes about 10 hours, the residue was dissolved in 1 ml of absolute ethanol, and the radioactivity was determined in a scintillation spectrometer for ^{14}C after the addition of 10 ml of liquid scintillation counting fluid (4 grams of PPO and 50 mg of POPOP in per liter of toluene), at an efficiency of approximately 70 %. The activity of PNMT in each tube is expressed as CPM/mg protein, and is plotted in Fig. 1-1 (p. 65).

In Fig. 1-1, the fractions showing highest PNMT activity were #34-#37, so that they were pooled, divided into small volumes and stored in the freezer (-20°C) for subsequent use.

C. DBH Assay.

Chemicals and Reagents. (1) Triton X-100 (octylphenoxypolyethoxyethanol), tyramine HCl, catalase (2x cryltallized from beef liver, suspended in water with 0.1 % thymol, activity 30,000-40,000 Sigma units/mg) were purchased from Sigma, St. Louis. (2) L-Ascorbic acid was a product of Nutritional Biochemicals Corporation, Cleveland, Ohio. (3) Disodium fumarate was purchased from K & K Laboratories, Plainview, New York. (4) Pargyline HCl (Eutonyl), which was a product of Abbott Laboratories, North Chicago, Illinois, was obtained through the courtesy of DR. A.O. Geiszler.

DBH was assayed by a modification of the procedure described by Molinoff et al. (1971). Sephadex column eluate, 50 μl in volume, was added

to a 15-ml test tube containing 150 λl of 0.1 % Triton X-100 in 0.01 M Tris buffer, pH 7.0. Twenty-five λl of $CuSO_4$ solution in appropriate concentration was added which would give optimal activity for the DBH preparation. The reaction was started by adding 100 λl of pre-mixed solution containing 25 λl of ascorbic acid solution (0.024 M, or 8.5 mg ascorbic acid in 1 ml of 0.1 M Tris, pH 5.5, fresh), 25 λl of fumarate solution (0.5 M, or 80 mg disodium fumarate in 1 ml of 0.1 M Tris, pH 5.5, fresh), 20 λl of pargyline solution (0.006 M, or 1.2 mg pargyline HCl in 1 ml of 0.1 M Tris, pH 5.5, fresh), 10 λl of 1 M Tris buffer, pH 5.5, 10 λl of catalase solution (1500 units) and 10 λl of tyramine solution (0.03 M, or 520 mg tyramine HCl in 100 ml of 0.01 N HCl solution). The total volume of the reaction mixture was 325 λl, with the cupric ion concentration at about 5 λmoles per liter. The reaction was run at 37°C in a Dubnoff metabolic shaking incubator. After 30 minutes the first step of the assay (DBH reaction proper) was stopped and the second step (PNMT reaction) was initiated by adding to each tube 100 λl of a mixture containing 20 λl of purified PNMT solution, 60 λl of 1 M Tris buffer, pH 8.6, 10 λl of EDTA solution (470 mg of disodium EDTA in 100 ml of water, pH 8.6) and 10 λl of ^{14}C-SAM (1.2 nmoles). The reaction was run in a total volume of 425 λl at 37°C for another 30 minutes, and was stopped by the addition of 0.5 ml of 0.2 M sodium borate buffer, pH 10. The rest of the assay procedure was carried out entirely the same as that for the PNMT assay (pp. 62-63) and the radioactivity of the final reaction product, ^{14}C-N-methyl-octopamine, was counted. The DBH activity in each tube was expressed as CPM/mg protein, and was plotted in Fig. 1-1 (p. 65).

In Fig. 1-1, the fractions showing highest DBH activity were #12-#15, so that they were pooled, divided into small volumes and stored in the freezer (-20°C) for subsequent use.

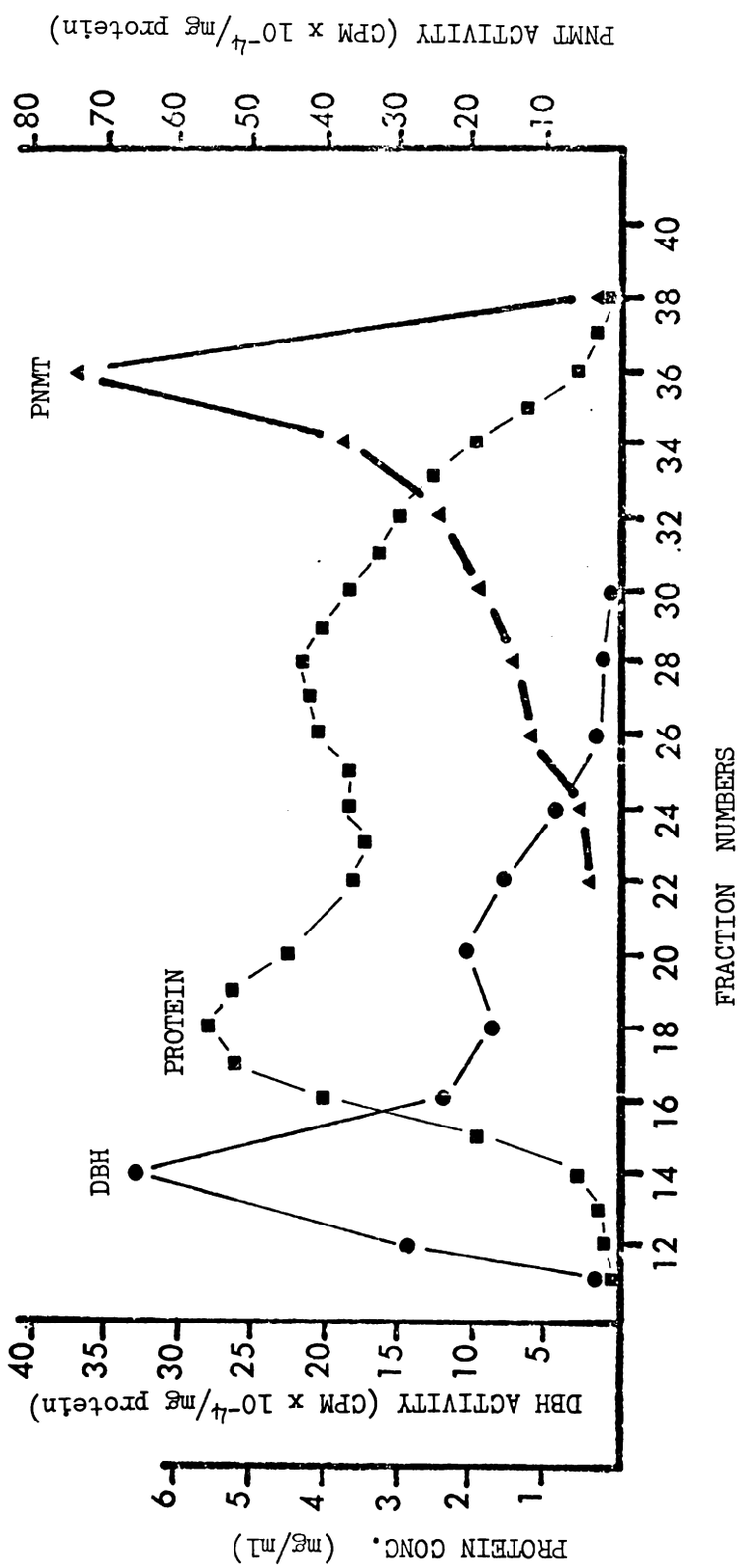


Fig. 1-1 Gel Chromatography of PNMT and DBH on Sephadex G-200.

In order to separate PNMT from DBH, 20 ml of concentrated protein solution are applied to a 3 x 80 cm column of Sephadex G-200. The protein are eluted in 8-ml fractions with 0.05 M Tris buffer, pH 7.4. Protein concentration is determined by reading the absorbance at 280 mμ/260 mμ.

DISCUSSION The proteins eluted from the column did not show distinct peaks. However, when fractions of the column eluate were assayed for PNMT and DBH activity, respectively, clear-cut separation of the two enzymes was achieved. The molecular weight for DBH was estimated to be about 290,000, while that of PNMT was about 32,000; hence it is easy to separate them by running through a column of Sephadex G-200 gel. The two enzyme preparations obtained by this procedure are only partially pure; nonetheless, the separation was adequate for our purpose. The PNMT would be used for catalyzing the second step of coupled reactions for assaying DBH activity, and the DBH could be used for studies on its biochemical properties.

==== C H A P T E R II - 2 ====

CHARACTERISTICS OF DBH REACTION : ROLE OF ASCORBIC ACID *

OBJECTIVE To investigate the presence of superoxide anion in the ascorbate-dependent DBH system so as to determine the role of ascorbate in this system, which has not been clearly defined.

INTRODUCTION As mentioned in Chapter I-6, the hydroxylation reaction catalyzed by dopamine-β-hydroxylase is aerobic in type, requiring molecular oxygen and an external electron donor. Thus DBH is classified as a "mixed function oxidase" (Kaufman and Friedman, 1965) and ascorbate has been shown to be essential for the enzymatic activity in vitro (Levin et al., 1960). Dopamine-β-hydroxylase is a copper-containing protein: the cupric ion in the enzyme has been shown to undergo cyclic reduction and oxidation during the catalytic reaction. Ascorbate is acting as a reducing agent and electron donor in the reaction and its function is presumably in the reduction of cupric ion in the enzyme molecule to cuprous form, which is catalytically active. Tryptophan-2,3-dioxygenase and protocollagen proline hydroxylase are other metal-requiring enzymes in which superoxide anions have been shown to be responsible for the conversion of oxidized metallic ions into active reduced form. By analogy it was speculated that superoxide anions may also be involved in the reductive reactivation of cupric ion in DBH, and the role of ascorbic acid may be in the generation of this free radical species. NADH-phenazine methosulfate, a non-enzymatic system which generates superoxide anions (Nishikimi et al., 1972), was

* In collaboration with Dr. T.Z. Liu.

chosen to be tested for its ability to substitute ascorbic acid in the DBH system. In contrast, some agents which remove superoxide from the system were also tested.

METHODS AND RESULTS

Chemicals. NADH (nicotinamide-adenine dinucleotide, reduced form, disodium salt) and phenazine methosulfate (P·MS) were products of Calbiochem, La Jolla, California. Nitro blue tetrazolium (NBT) was purchased from Sigma, St. Louis.

Beef adrenal DBH was prepared by the method described in Chapter II-1. When standard amounts of octopamine were run along with this partially purified DBH preparation, it was estimated that the DBH catalyzed the formation of 53 nmoles of octopamine from tyramine substrate per mg protein per 30 minutes at 37°C. Protein concentration was measured by the method of Lowry et al. (1951).

DBH activity was measured by the two-step procedure described in Chapter II-1 with minor modification. At the first stage, the total reaction mixture in 375 μl contained: 50 μl of 0.024 M ascorbic acid (or NADH-P·MS solution in appropriate concentration), 25 μl of 0.5 M fumarate, 20 μl of 0.006 M pargyline, 10 μl of catalase (1500 units), 10 μl of 1 M Tris (pH 5.5), 10 μl of 0.03 M tyramine, 25 μl of 0.1 mM $CuSO_4$, 25 μl of NBT in appropriate concentration (or water), and 200 μl of DBH solution containing 110-310 μg of protein. An equivalent amount of water was added to substitute the deleted cofactor. The reaction was started and proceeded to the end entirely the same as it was described in Chapter II-1.

A. Effect of NADH-P·MS System on DBH Activity in the Absence of Ascorbate.

As indicated in Table 2-1, complete DBH activity occurred when ascorbate was present in the reaction mixture. Elimination of ascorbate lowered the enzyme activity to 19 % of the control. However, when NADH-P MS was added to the reaction mixture in place of ascorbate, DBH activity was comparable to that observed in the controls. Since NADH-P·MS has been demonstrated to generate superoxide, these data suggest that the role of ascorbate in the DBH system is in the generation of superoxide anions (O_2^-).

TABLE 2-1 Effect of NADH-Phenazine Methosulfate (NADH-P·MS) System on DBH Activity in the Absence of Ascorbate.

Addition or Deletion	DBH Activity[1] (% of control)
Complete system[2]	100
− Ascorbic acid	19
− Ascorbic acid, + NADH-P·MS[3]	110
− Enzyme[4], − Ascorbic acid, + NADH-P·MS[3]	0.5

1) Results were the average values of at least two determinations.
2) Ascorbic acid was present in the reaction mixture.
3) The final concentrations of NADH and P·MS were 0.15 mM and 0.30 mM, respectively.
4) Boiled enzyme.

The effect of varying the concentration of phenazine methosulfate on DBH activity in the absence of ascorbate was examined, and the data are presented in Fig. 2-1. It is evident that increased DBH activity was observed with increasing concentration of P·MS. Near maximum activity

was obtained at a concentration of 0.15 mM.

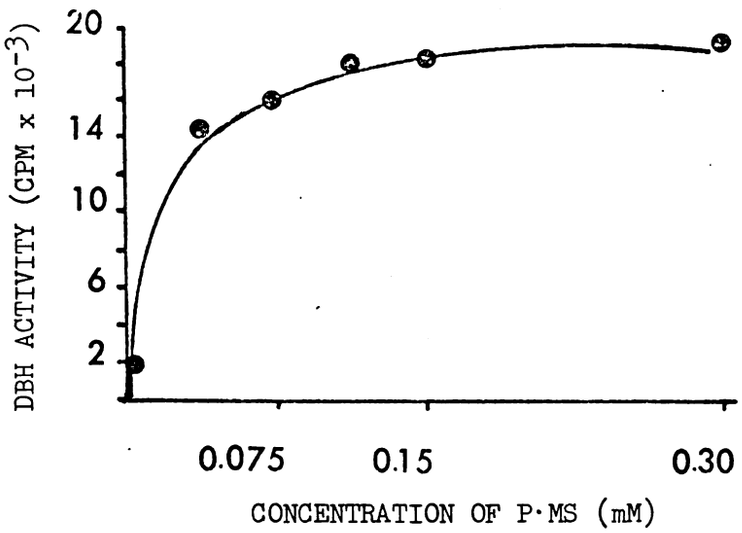


Fig. 2-1 Effect of Phenazine Methosulfate (P·MS) on DBH Activity.

Incubation was carried out in the absence of ascorbic acid. Each tube contained 0.15 mM of NADH, and the amount of enzyme was 310 μg per tube.

B. Effect of Nitro Blue Tetrazolium (NBT) on DBH Activity.

Nitro blue tetrazolium has been shown to be a competitor for free radicals (Beauchamp and Fridovich, 1971), and will remove them from the reaction system. As evident from Fig. 2-2, ascorbate-dependent DBH activity was greatly inhibited by this superoxide scavenger.

It was of interest to see whether the activation of DBH by NADH-P·MS in the non-ascorbate system was also inhibited by NBT. As indicated in Fig. 2-3, NBT progressively inhibited NADH-P·MS -supported DBH activity in the non-ascorbate system. These results substantiate the role of

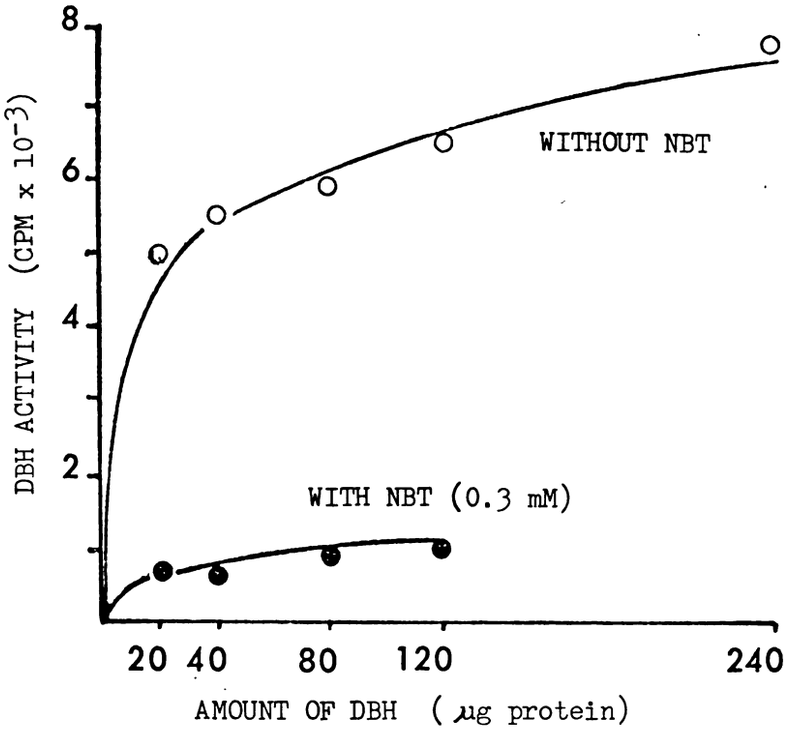


Fig. 2-2 Effect of Nitro Blue Tetrazolium (NBT) on DBH Activity in the Reaction Mixture Containing Ascorbate.

NADH-P·MS as the generator of superoxide, and further suggest that a free radical intermediate is involved in the DBH reaction.

C. Effect of Superoxide Dismutase on DBH Activity.

Superoxide dismutase which catalyzes the dismutation of O_2^- to hydrogen peroxide was purified from fresh bovine erythrocytes according to the procedure of McCord and Fridovich (1969). DBH activity in this experiment was assayed by a simpler procedure described in next chapter (p. 75). As shown in Fig. 2-4, DBH activity was markedly inhibited when various amounts of superoxide dismutase were incubated in the reaction mixture. This

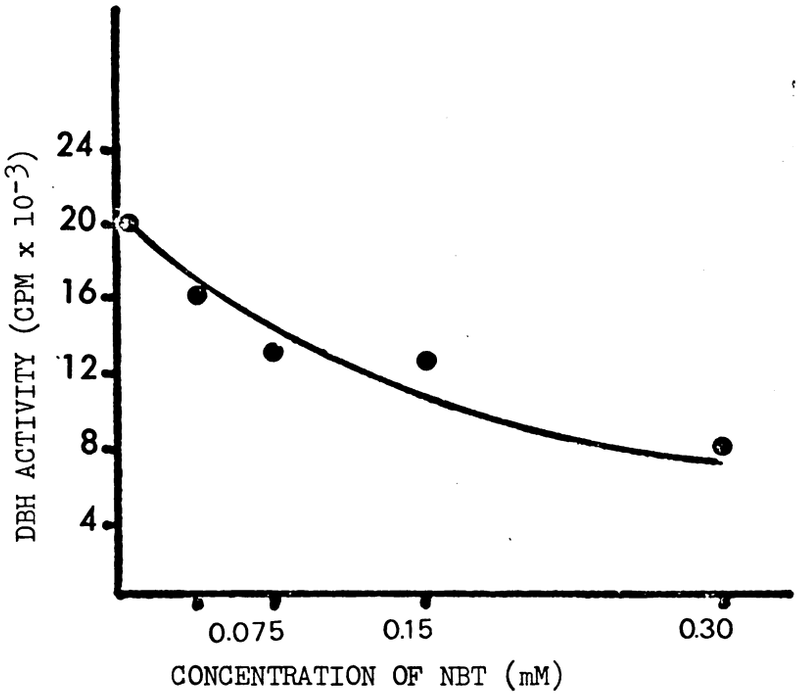


Fig. 2-3 NBT Inhibition of DBH Activity Supported by NADH-P·MS System in the Absence of Ascorbate.

The concentrations of NADH and phenazine methosulfate used for each tube were 0.15 and 0.30 mM, respectively.

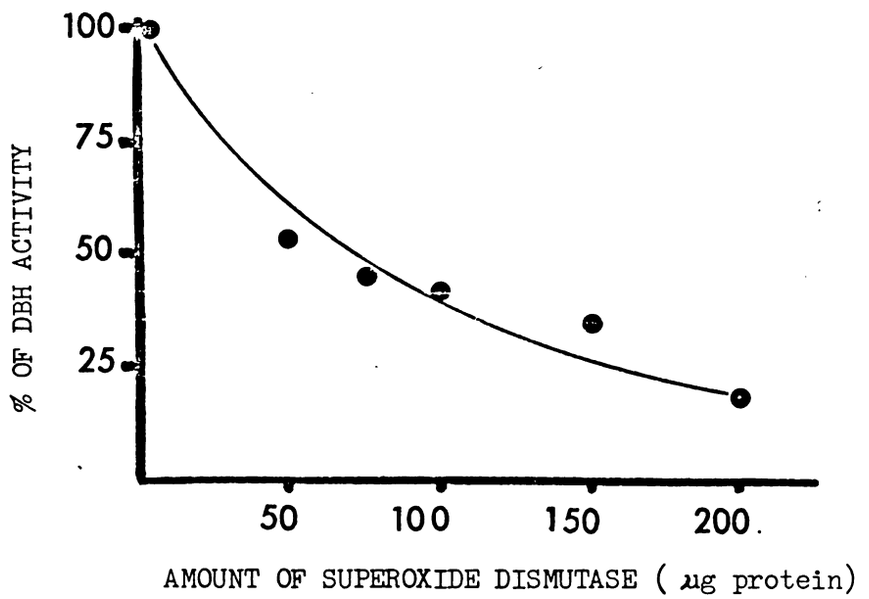


Fig. 2-4 Inhibition of Ascorbate-Dependent DBH Activity by Superoxide Dismutase.

finding confirms that superoxide anion is required for the DBH activity.

DISCUSSION The present studies confirm that ascorbate is essential for the DBH activity, demonstrate that ascorbate can be replaced by a NADH-phenazine methosulfate system which generates superoxide anions, and that nitro blue tetrazolium or superoxide dismutase can inhibit the DBH activity by removing superoxide free radicals from the system. These findings suggest that O_2^- is an essential intermediate for the enzymatic reaction, and that the role of ascorbate is probably in the generation of O_2^-. The superoxide generated in the system may act to convert the oxidized form of copper ion into reduced form (Cu^+), which is regarded as catalytically active.

==== C H A P T E R II - 3 ====

THE EFFECT OF HYDRALAZINE ON DBH ACTIVITY IN VITRO *

OBJECTIVE To study the effect of a chelating agent, hydralazine, on the activity of dopamine-β-hydroxylase *in vitro*.

INTRODUCTION DBH is a copper-containing protein, and many chelating agents, such as diethyldithiocarbamate (DDC), KCN, α,α'-dipyridyl, EDTA, etc., have been shown to inhibit the enzyme activity *in vitro* and *in vivo* (Green, 1964; Goldstein et al., 1964).

The drug hydralazine (L-hydrazinophthalazine hydrochloride, Apresoline) has been used for many years as an antihypertensive agent. However, little is known about the precise mechanism(s) of its antihypertensive action. Considering the close correlation between blood pressure and plasma norepinephrine levels in patients with essential hypertension (Louis et al., 1973) and the ability of hydralazine to chelate metallic ions (Schroeder, 1959), we considered the possibility that hydralazine may inactivate DBH by binding the cupric ions in the nezyme, leading to decreased formation of norepinephrine which may account in part for the blood-pressure lowering action of hydralazine. Using the partially purified DBH from beef adrenal medulla, we stidied the effect of hydralazine on the activity of DBH *in vitro*.

METHODS AND RESULTS

Chemicals. Hydralazine HCl was purchased from Sigma, St. Louis. I side chain-1,2-^{3}HI Tyramine hydrochloride (specific activity 5.5 Ci/mmole)

* In collaboration with Dr. T.Z. Liu.

was purchased from Amersham/Searle Corporation, Arlington Heights, Illinois.

Unlike most of the experiments in this dissertation, the DBH activity in this particular study was measured by a simpler procedure developed by us. The principle of the assay was based on the conversion by DBH of [side chain-1,2-^{3}H] tyramine to the β-hydroxylated amine, with simultaneous release of a tritium to form tritiated water in the reaction mixture. The quantity of tritiated water formed was measured as an index for DBH activity.

The standard incubation mixture contained tyramine substrate in 1.0 M Tris buffer, pH 5.5 (500 μg cold plus 5 x 10^{6} CPM hot tyramine per ml), 200 μl; catalase, 100 μl (5,000 units); ascorbic acid (5.6 mM), 200 μl; disodium fumarate (22 mM), 200 μl; pargyline HCl (20 mM), 50 μl; $CuSO_4$ (0.72 mM), 50 μl; DBH preparation, 50-400 μg in 200 μl; and hydralazine in appropriate concentrations, 100 μl. An equivalent volume of water was added to replace the deleted component in the system. The total volume of the mixture was 1.1 ml, and the reaction was run for 30 minutes at 37°C. After termination of the reaction by adding 0.1 ml of 50 % trichloroacetic acid, the tritiated water was removed from the reaction mixture by vacuum distillation and collected in a test tube. Aliquots of 0.5 ml were mixed with 10 ml of aqueous scintillation fluid and counted for tritium. Samples of boiled enzyme preparation were used as blank. The low blank radioactivity (ranging from 100 to 300 CPM) indicated that the nonspecific tritium-hydrogen exchange during the reaction was negligible.

A. Effect of Cupric Ions on Partially Purified Beef Adrenal DBH.

It is well documented that full activity of DBH is reduced in various tissues by endogenous inhibitors which can be neutralized by appropriate concentrations of cupric ion. Therefore, it is important to establish the

optimal copper concentration required to inactivate the endogenous inhibitors before any experiment on DBH is carried out. As indicated in Fig. 3-1, the narrow range of copper concentrations required for the optimal enzyme activity verifies the importance of this quantitation. Enzyme protein concentration used for this experiment was 400 μg per tube. The optimal copper concentration required under this condition was estimated to be 3.6×10^{-5} mole per liter of reaction mixture.

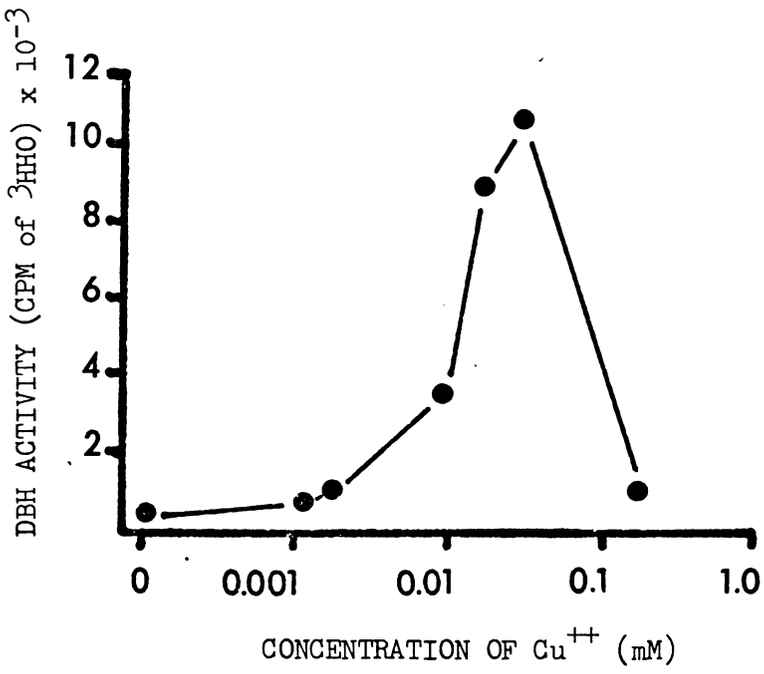


Fig. 3-1 Effect of Cupric Ion Concentration on Partially Purified DBH.

There was 400 μg of enzyme in 1.1 ml of reaction mixture. Each point represents the average of two separate assays.

B. Effect of Hydralazine on DBH Activity.

As shown in Fig. 3-2, when partially purified beef adrenal DBH was incubated with various concentrations of hydralazine along with appropriate

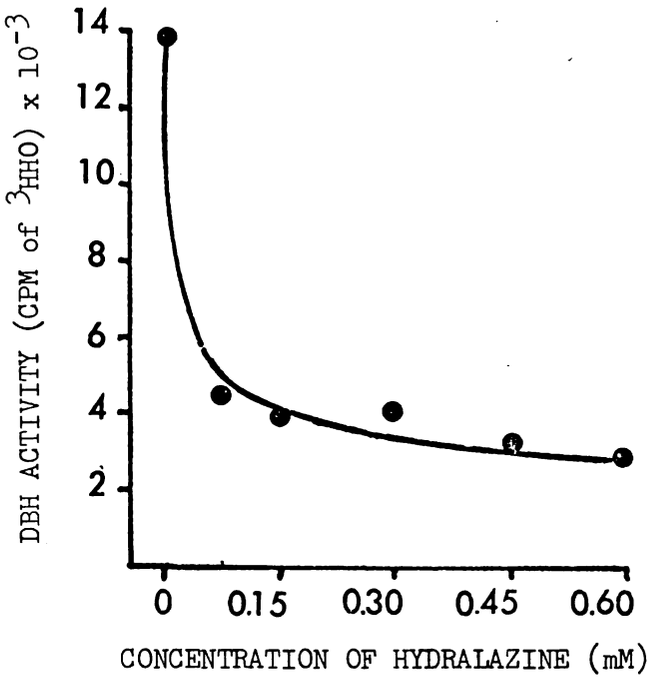


Fig. 3-2 Effect of Hydralazine on DBH Activity In Vitro.

cofactors, inhibition of enzyme activity occurred.

Fig. 3-3 shows that, in the absence of hydralazine, DBH activity rises linearly with the increased amount of enzyme. However, in the presence of hydralazine (0.1-0.2 mM), the enzyme activity was reduced to 10 % of the control.

The Lineweaver-Burk plot (substrate^{-1} vs velocity^{-1}) was examined in the absence and presence of hydralazine. As indicated in Fig. 3-4, the two lines share a common intercept on the abscissa. This suggests that the inhibition of DBH by hydralazine was noncompetitive in nature. Therefore, the drug does not seem to interfere with the substrate binding site of the enzyme.

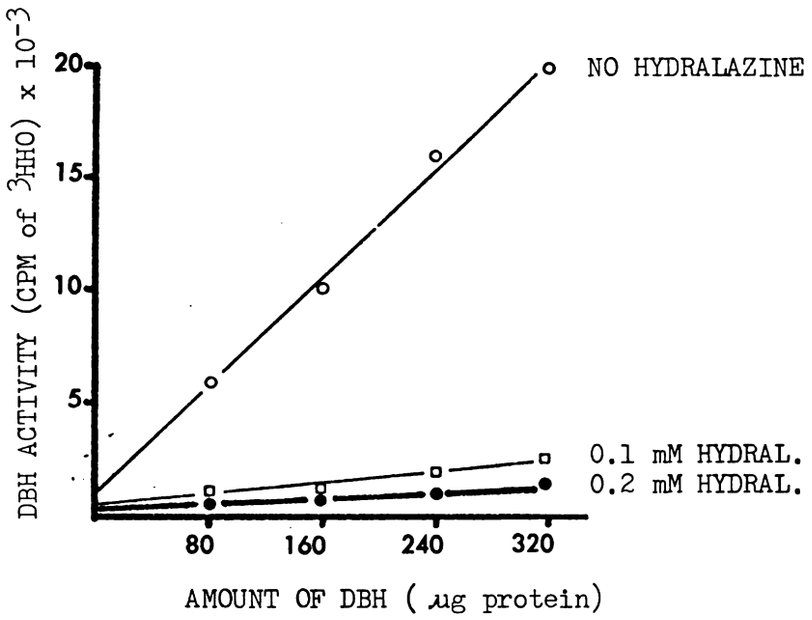


Fig. 3-3 Effect of DBH Concentration on DBH Activity in the Absence or Presence of Hydralazine.

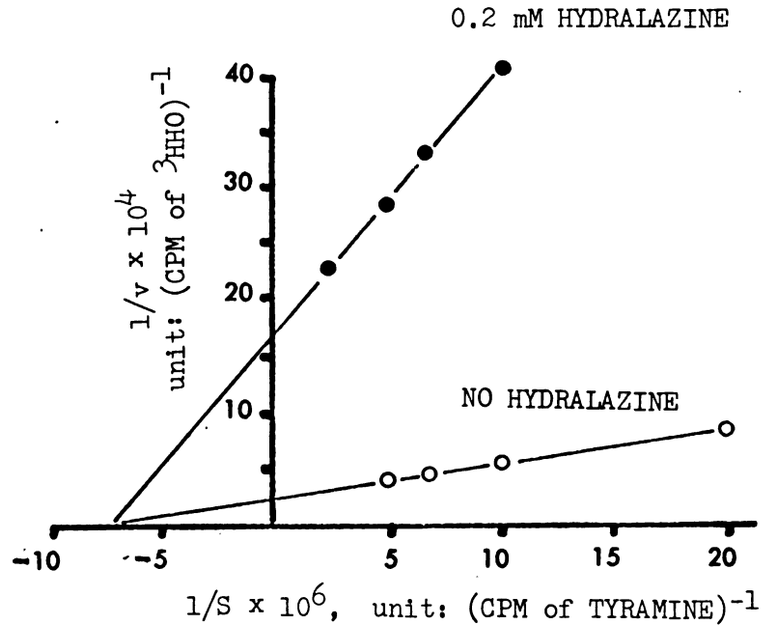


Fig. 3-4 Lineweaver-Burk Plot of Beef Adrenal DBH in the Absence or Presence of Hydralazine.

It was of interest to see whether the drug and DBH were competing for an essential constituent directly involved in the enzymic action. Hence, a double reciprocal plot of the initial velocity against enzyme protein concentration in the absence or presence of hydralazine was constructed. As shown in Fig. 3-5, all three lines shared a common intercept on the abscissa, indicating that the drug and DBH do not compete for an active component required for the enzyme catalysis.

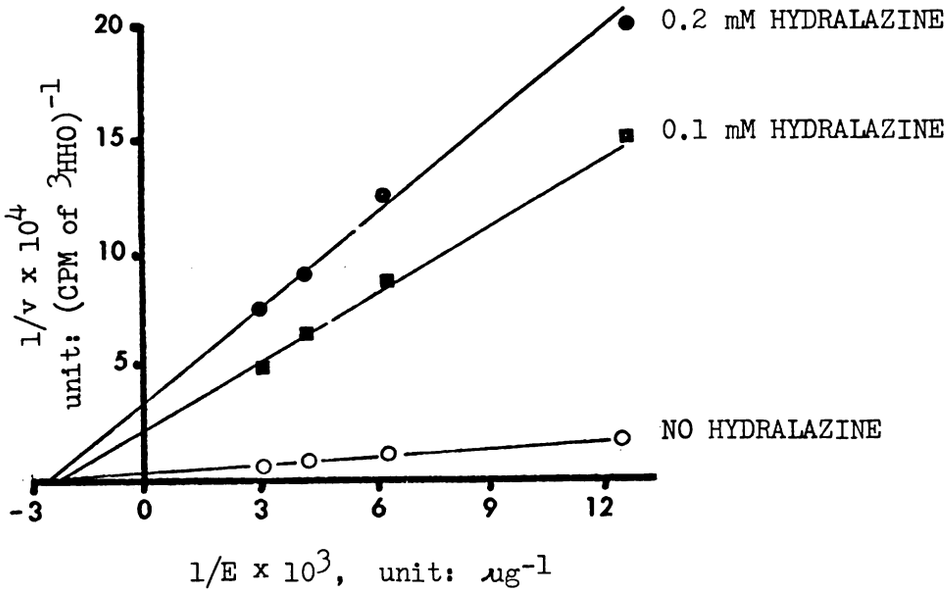


Fig. 3-5 Double Reciprocal Plot of the Initial Velocity against Enzyme Protein Concentration in the Absence or Presence of Hydralazine.

C. Effect of Cupric Ions on the Reversal of DBH Activity Inhibited by Hydralazine.

Considering the obligatory requirement of copper for DBH activity and the ability of hydralazine to chelate metal ions, we directed our attention to the nature of hydralazine-induced inhibition of DBH activity with

special reference to the chelation of copper in the system by hydralazine. In Fig. 3-6, various concentrations of cupric ion were added back to the DBH system after it was incubated with hydralazine for 10 minutes. The dose-dependent reversal of the enzyme inhibition by cupric ions was evident. These data strongly suggest that the formation of a chelating complex between hydralazine and copper ions, which subsequently leads to the inactivation of DBH or the disinhibition of endogenous inhibitors, is responsible for the decreased DBH activity in vitro.

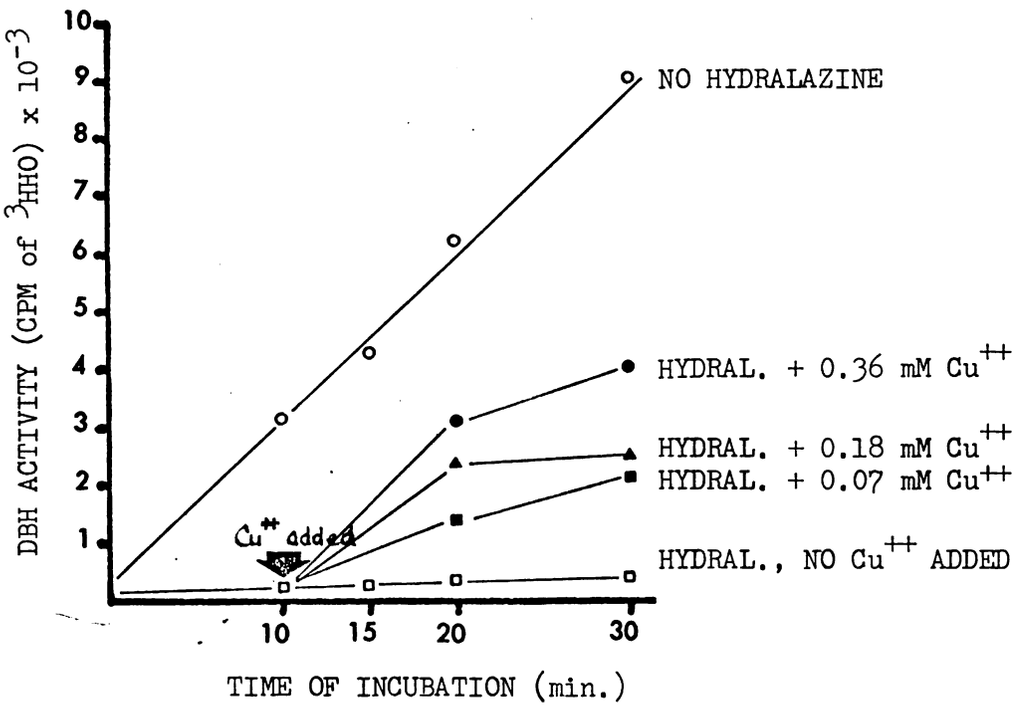


Fig. 3-6 Effect of Cupric Ions on the Reversal of DBH Activity Inhibited by Hydralazine (1.0 mM).

DISCUSSION The partially purified bovine adrenal DBH was contaminated by endogenous inhibitors which could be neutralized by appropriate concentration of cupric ions. When hydralazine was added to the enzyme prepara-

tion in vitro along with other cofactors, the hydroxylase activity was inhibited. This inhibition was shown to be noncompetitive with the substrate for the same binding site in the enzyme molecule, nor was it through competition for an essential constituent directly involved in the optimal catalytic action of the enzyme. Rather, the drug-induced inhibition of the enzyme activity was reversed by adding various concentrations of cupric ion to the system. These data suggest that the drug inhibits DBH activity by chelating cupric ions which are required for the inactivation of endogenous inhibitors present in the enzyme preparation.

It is possible that hydralazine inhibits DBH activity in vivo when it is given to intact animals. The decrease in norepinephrine content in certain tissues after hydralazine has indeed been demonstrated. Bygdeman and Stjärne (1959) found decreased levels of catecholamines in the heart and adrenals of rats after two doses of 5 mg/kg dihydralazine, but subsequently they could not reproduce these findings satisfactorily (Bygdeman and Stjärne, 1960). Linet et al. (1969) observed a fall in the endogenous norepinephrine of rat heart after daily injection of 20 mg/kg of dihydralazine for two days, whereas that of the brain and adrenals remained unchanged. The time course of the decrease in norepinephrine content in rat heart after dihydralazine revealed that the maximum effect was attained at 4 hours which subsequently returned to normal levels at 6 hours, indicating a compensatory increase in norepinephrine synthesis.

Hydralazine was also shown to inhibit dopa decarboxylase and histidine decarboxylase (Werle et al., 1955). It is difficult, however, to determine to what extent the inhibition of dopa decarboxylase and dopamine-β-hydroxylase, with concomitant decrease in cardiac norepinephrine, contributed to the blood-pressure lowering action of hydralazine. Although we have clearly demonstrated the inhibition of DBH activity in vitro by hydralazine,

==== C H A P T E R II - 4 ====

STANDARDIZATION OF ASSAY PROCEDURE FOR MEASURING DBH ACTIVITY IN REGIONS OF RAT BRAIN

OBJECTIVE (1) To dissect specific regions of rat brain.

(2) To homogenize the brain tissue and set up DBH assay for brain homogenate.

(3) To determine the optimal copper concentration for brain homogenate.

(4) To identify the reaction product by thin-layer chromatography.

(5) To study the intra- and interassay variation of the assay.

INTRODUCTION Although the presence of dopamine-β-hydroxylase in the brain has been described (Udenfriend and Creveling, 1959), previous methods have not been sensitive nor specific enough to provide an analysis of the changes in DBH activity after physiological manipulation. With the development of a specific and highly sensitive assay for DBH in tissue homogenates (Molinoff et al., 1971), DBH in regions of rat brain has been studied during development (Coyle and Axelrod, 1972), after drug treatment (Reis and Molinoff, 1972) and after manipulation of different endocrine systems (Kizer et al., 1974). I set up and standardized this assay procedure to measure DBH activity in specific regions of rat brain. I chose to study the hypothalamus which is the final common site for neuroendocrine integration and is a region rich in noradrenergic nerve terminals. I also studied the brain stem, where the perikarya of noradrenergic neurons are located, and the hippocampus, where glucocorticoids are concentrated. The hippocampus is believed to be an important site of glucocorticoid action in the brain (McEwen et al., 1969; Gerlach and McEwen, 1972).

METHODS AND RESULTS

A. Dissection of Rat Brain Regions.

Male Sprague-Dawley rats were obtained from Simonsen Laboratory Inc., Gilroy, California and were used throughout this research, unless it was specified in only a few instances. The rats, weighing 150-200 grams, were sacrificed by decapitation at the level of foramen magnum, and the skull vault was cut to expose the brain. After removing the cerebellum, the rest of the brain was taken out from the cranial cavity. The brain stem, which includes pons and medulla oblongata, was separated from cerebral hemispheres and put on dry ice (Figs. 4-1 and 4-3). After cutting the cortical layer, the hippocampi were dissected out according to the diagrams in Figs. 4-1 and 4-2, and were placed together on dry ice. After the rest of the brain was frozen, the hypothalamus was dissected out with a razor blade and kept on dry ice (Figs. 4-1, 4-3 and 4-4).

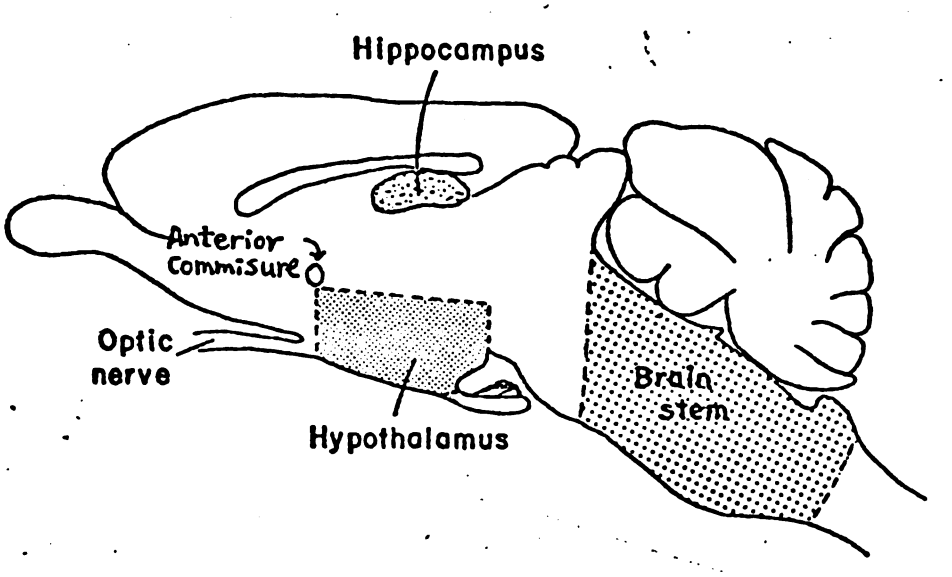


Fig. 4-1 Mid-Sagittal Section of Rat Brain.
(Modified from McEwen et al., 1969.)

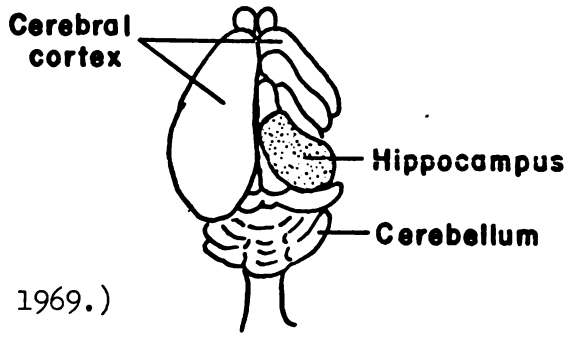


Fig. 4-2 Rat Brain with cortical layer of right cerebral hemisphere removed to show the hippocampus. (Modified from McEwen et al., 1969.)

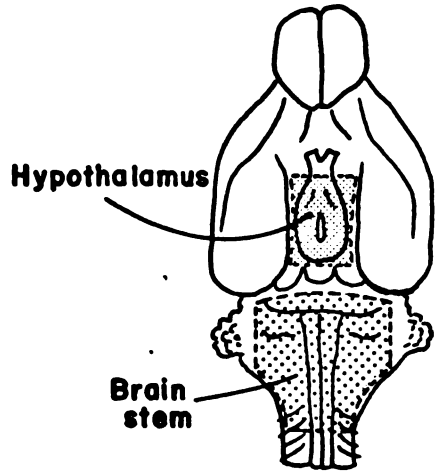


Fig. 4-3 Inferior surface of rat brain, showing hypothalamus and brain stem. (Modified from Glowinski and Iversen, 1966.)

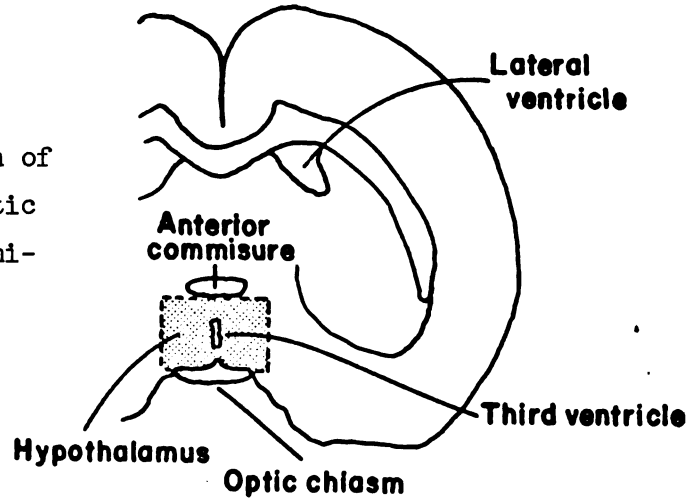


Fig. 4-4 Coronal section of rat brain through the optic chiasma and anterior commisure, showing the third ventricle and the hypothalamus.

B. Homogenization of Brain Tissue and Assay of DBH Activity.

Brain tissue was weighed and homogenized in 40 vol. (w/v) of ice-cold 0.01 M Tris-HCl buffer containing 0.1 % (w/v) Triton X-100, pH 7.0. The homogenates were centrifuged at 10,000 x g for 15 minutes. The supernatant fluid was decanted, kept in a refrigerator at 4°C, and assayed within 24 hours. DBH activity in the supernatant of brain homogenate was measured by the coupled radioenzymatic procedure described in Chapter II-1 (pp. 63 -64) with minor modifications. For the DBH reaction proper, the total reaction mixture in 325 µl contained: 200 µl of brain homogenate (equivalent to 5 mg of brain tissue), 25 µl of $CuSO_4$ solution (24.35 grams of $CuSO_4 \cdot 5\ H_2O$ in 100 ml water) to give a final cupric concentration of 75 µM, and 100 µl of pre-mixed solution containing those components at pH 5.5 as described in Chapter II-1 (p. 64). The rest of the reaction procedure was carried out entirely the same as it had been described (p. 64). For the second step, 30 µg of PNMT and 1.2 nmoles of ^{14}C-SAM were added to each tube.

Boiled supernatant of brain homogenate was run as the blank. Standard amounts of octopamine hydrochloride were run along with each assay for the plotting of a standard curve. The amount of DBH activity in the brain tissue was expressed as nanomoles of octopamine formed per gram of brain per hour (nmoles oct./gm/hr). In a typical assay, the radioactivity in the blank tubes were around 500 CPM, while those for the sample tubes were between 4,000 and 8,000 CPM.

C. Determination of Optimal Cupric Ion Concentration for Brain Homogenate.

The DBH activity of 1:40 (w/v) homogenates of various brain regions was assayed in the presence of different concentrations of cupric ion. As shown in Table 4-1, the optimal cupric ion concentration for three brain

regions-hypothalamus, brain stem and hippocampus- is 75 μM in each case.

TABLE 4-1 Effect of Different Cupric Ion Concentrations on DBH Activity in Regions of Rat Brain.

All values are the means ± S.E.M., n = 4.

Cu^{++} Concentration (μM)	D B H Activity (nmoles oct./gm/hr)		
	Hypothalamus	Brain Stem	Hippocampus
0	0	0	0
50	135.8 ±17.6	138.2 ±26.5	23.2 ±2.1
75	279.9 ± 2.6	177.5 ±14.3	98.1 ±7.4
100	258.1 ±13.0	113.7 ±14.8	80.6 ±0.4
125	222.9 ±15.9	70.0 ±10.0	
150	137.8 ±20.1		

D. Identification of DBH-PNMT Reaction Products by Chromatography.

Chemicals. N-Methyl-octopamine (DL-synephrine) and L-epinephrine were purchased from Sigma, St. Louis. N-Methyl-tyramine was kindly supplied by Dr. J.L. McLaughlin at the School of Pharmacy and Pharmaceutical Sciences, Purdue University.

As described in Chapters I-6 and II-1, when tyramine was used as the substrate the β-hydroxylated product of DBH reaction was octopamine, which

was rendered radioactive through N-methylation catalyzed by PNMT with S-adenosyl-methionine [^{14}C-methyl] as the methyl donor. The final reaction product for the coupled enzymatic reaction, therefore, was N-methyl [^{14}C] - octopamine. To identify the radioactive products extracted after the reaction, thin-layer chromatography was carried out on pre-coated TLC plates (Silica Gel 60, size 5 x 20 cm, 0.25 mm in thickness, EM Laboratories Inc., Eimsford, New York). The solvent systems used were either a mixture of ethanol (96 %) and NH_4OH (25 %) in the proportion of 80 : 20, or a mixture of n-butanol, glacial acetic acid and water in the proportion of 40:10:50 (Table 94 of Stahl and Schorn, 1969). After development, the plates were sprayed with iodine solution to localize the authentic amines. The silica gel was scraped out from the plate in 1 x 1 cm squares which were collected in counting vials containing 0.5 ml of NCS (Amersham/Searle), a surface-active organic base. After one hour, 1 ml of ethanol and 10 ml of counting fluid (PPO and POPOP in toluene, see p. 63) were added to each vial, and the radioactivity was determined for ^{14}C.

The results of thin-layer chromatography of the radioactive products of DBH-PNMT reaction are shown in Fig. 4-5 and Fig. 4-6, using different solvent systems. The only peak of radioactivity which differs between the experimental and blank samples has the same R_f as authentic N-methyl-octopamine. When exogenous octopamine was incubated in the presence of PNMT, the radioactive product was also N-methyl-octopamine. The samll radioactive peak present in the blank has the same R_f as N-methyl-tyramine, the product which was formed from N-methylation of the non-β-hydroxylated amine, tyramine. These findings indicate that this coupled enzymatic reaction is rather specific in the sense that it leads to the formation of a well defined chemical species, N-methyl-octopamine. The radioactivity of this final product can be determined to express DBH activity in the brain.

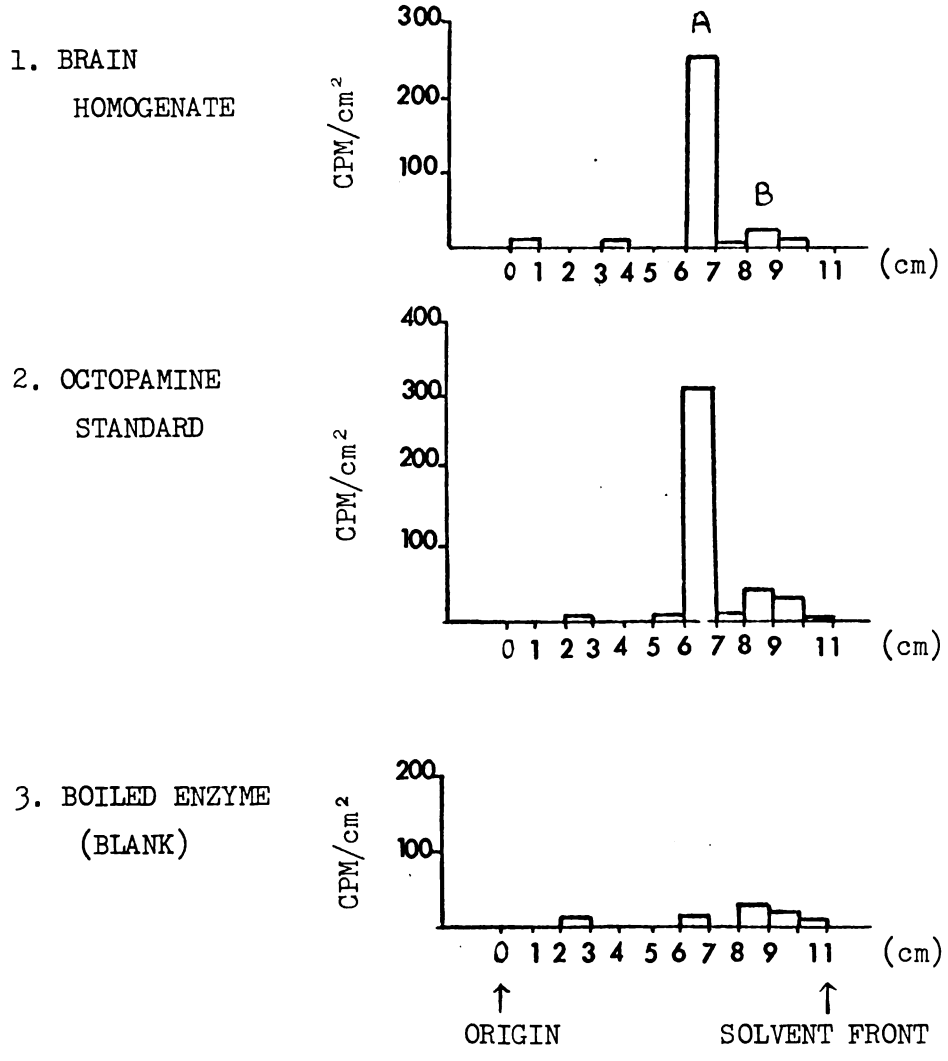


Fig. 4-5 Thin-Layer Chromatography of the Radioactive Products of DBH-PNMT Assay.

The abscissa represents distance from the origin of the solvent.
The solvent system was ethanol (96 %) : NH_4OH (25 %) = 80 : 20.
The authentic amines used were: A = N-methyl-octopamine,
B = N-methyl-tyramine.

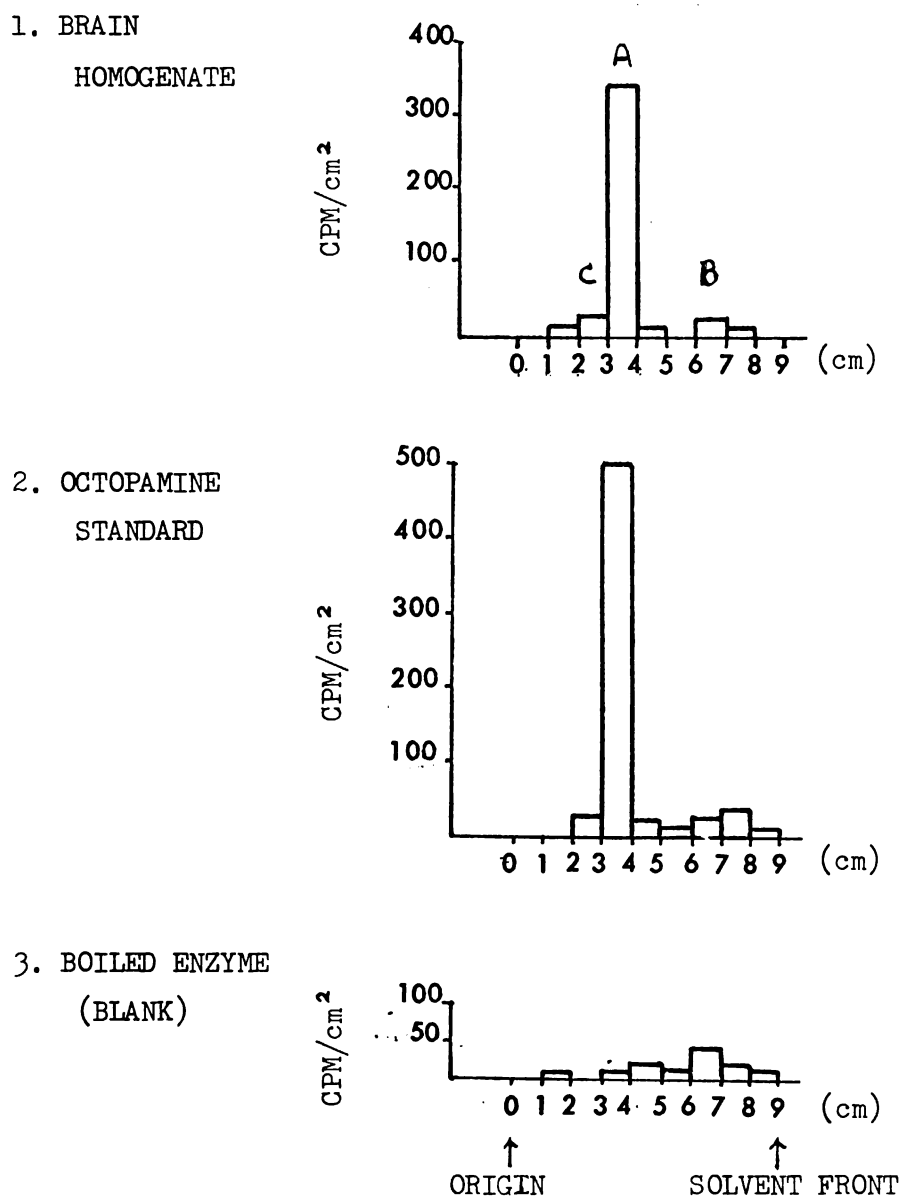


Fig. 4-6 Thin-Layer Chromatography of the Radioactive Products of DBH-PNMT Assay.

The abscissa represents distance from the origin of the solvent. The solvent system was n-butanol:glacial acetic acid:water = 40:10:50. The authentic amines used were: A = N-methyl-octopamine, B = N-methyl-tyramine, C = L-epinephrine.

E. Formation of N-Methyl-Octopamine by PNMT : Linearity of the Assay.

Standard amounts of exogenous octopamine were incubated in the presence of PNMT. As shown in Fig. 4-7, the radioactivity incorporated into N-methyl-octopamine after 30 minutes is directly proportional to the amount of octopamine added. This is also true when different amounts of PNMT were added to catalyze the reaction. Therefore, in the presence of more than 10 µg of PNMT, the reaction is linear for 25 to 200 ng of octopamine hydrochloride. The brain samples were diluted in such a way (1:40) that the amounts of octopamine formed in the DBH reaction proper fell in this range. In order to assure the formation of maximal amounts of N-methyl-octopamine, the subsequent DBH-PNMT reactions were run in the presence of 30 µg of PNMT, which gave rise to definite linear reactions.

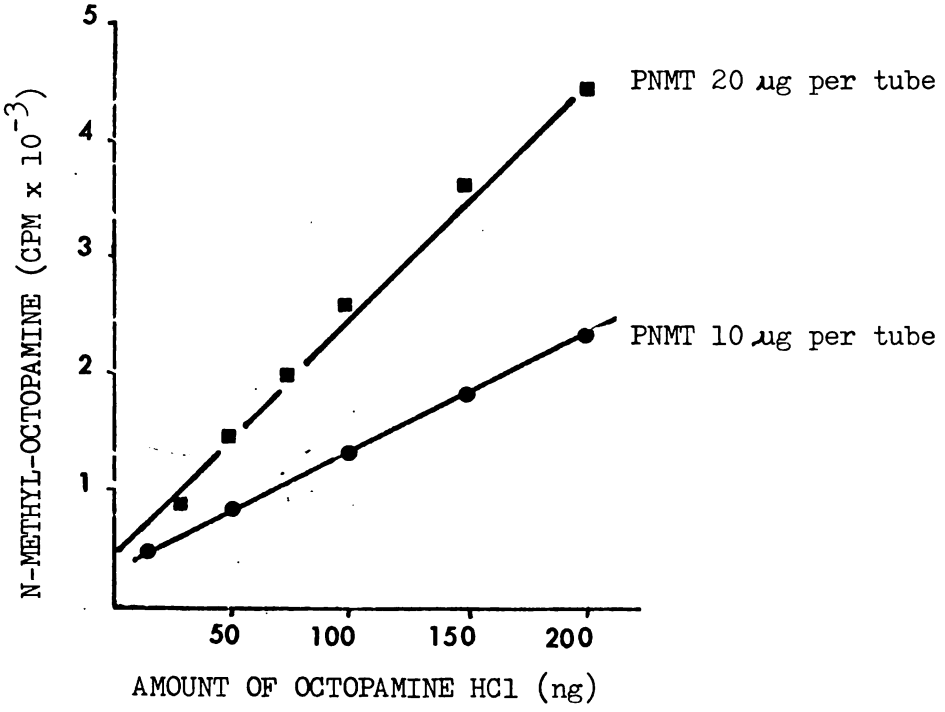


Fig. 4-7 Formation of N-Methyl-Octopamine by PNMT after 30 Minutes. The effect of increasing amounts of octopamine or PNMT on the formation of N-methyl-octopamine was determined in the presence of boiled brain homogenate. The cofactors were also present in their usual concentrations.

F. Intra- and Interassay Variation of DBH Assay.

Aliquots from a single pool of rat brain stem homogenate were run in a single batch assay to determine the DBH activity in each tube. DBH activity for 8 determinations is shown in Table 4-2, with a mean of 214.3 nmoles oct./gm/hr, and a standard error of mean at 3.7. The 95.5 % confidence interval of the DBH activity for that brain stem is 193.3 - 235.3 nmoles oct./gm/hr. It is evident that there is a small intra-assay variation for the DBH assay. An additional fact indicating the reproducibility of the assay is the similarity of the counts for duplicate determinations.

TABLE 4-2 Intra-assay Variation of DBH Assay.

Eight determinations from a single pool of rat brain stem homogenate, run in a single assay. DBH activity is expressed as nmoles oct./gm/hr.

227.2, 204.7, 211.2, 209.1, 226.7, 223.6, 212.9, 199.2 ;

Mean = 214.3, S.D. = 10.5, S.E.M. = 3.7 ;

95 % confidence interval = mean ± 2 S.D. = 193.3~235.3 .

The interassay variation was tested by running aliquots from a single pool of brain stem homogenate in different batches of assay. The seven determinations run in seven different assays during a period of 40 days are shown in Table 4-3, with a mean of 217.3 nmoles oct./gm/hr, and a standard error of mean at 3.6. The small coefficient of variation (4.3 %) again indicates that the assay for DBH activity is accurate and reproducible.

TABLE 4-3 Interassay Variation of DBH Assay.

Seven determinations for samples from a single pool of brain stem homogenate, run in seven different assays during a period of 40 days.
DBH activity is expressed as nmoles oct./gm/hr.

226.8, 227.9, 221.6, 211.0, 217.3, 216.2, 200.5 ;

Mean = 217.3, S.D. = 9.5, S.E.M. = 3.6 ;

Coefficient of variation = S.D./mean = 9.5 / 217.3 = 4.3 % .

G. Effect of Chemical Sympathectomy by 6-Hydroxydopamine (6-OHDA) on DBH Activity in the Brain.

6-Hydroxydopamine (6-OHDA) induces a specific degeneration of peripheral sympathetic nerve terminals with a marked depletion of norepinephrine. Its intracerebral or intraventricular injection also causes a destruction of adrenergic neurons with a decrease in the norepinephrine concentration and to a lesser extent in the dopamine concentration in the brain (for review, see Thoenen, 1972b).

In order to confirm the location of DBH in noradrenergic neurons and to test the specificity of the DBH assay, DBH activity in rat hypothalamus and brain stem was determined after destruction of noradrenergic neurons by centrally administered 6-OHDA. Male Sprague-Dawley rats, weighing about 300 grams, were given 6-OHDA hydrobrimide, 200 µg/day for 2 days, in a volume of 10 µl into the third ventricle through a chronically implanted cannula (Chapter II-11; Cuello et al., 1974). An equimolar quantity of sodium bromide was given to some rats as the controls. Drugs were dissolved in normal saline containing 0.1 % ascorbic acid. Rats were sacrificed

24 hours after the last injection.

As shown in Table 4-4, 6-OHDA produced a marked fall (about 60 %) in the activity of DBH in the hypothalamus and brain stem. The injection of sodium bromide alone seemed to lower the enzyme activity slightly. The findings that 6-hydroxydopamine, when given intraventricularly, caused a marked decrease in DBH activity in both the terminals and perikarya of noradrenergic neurons in the brain, are consistent with that of Reis and Molinoff (1972), and provide indirect evidence that the assay procedure for DBH is relatively specific and measures primarily the amount of DBH present in the noradrenergic neurons in the brain.

TABLE 4-4 Effect of Chemical Sympathectomy with 6-Hydroxydopamine (6-OHDA) on DBH Activity in the Brain.

DBH activity is expressed as nmoles oct./gm/hr.
All values are means ± S.E.M.

Treatment	No. of Animals	DBH Activity (nmoles/gm/hr) Hypothalamus	Brain Stem
Vehicle (NaBr)	6	218.9 ±18.2	122.8 ± 9.8
6-OHDA	7	95.1 ±10.6	44.0 ±14.3

H. Effect of Renal Denervation on DBH Activity in Dog Renal Cortex.

The specificity of DBH assay was further checked by studying the change in DBH activity in dog renal cortex after denervation. Cortical tissue was dissected from dog kidney and homogenized in 10 vol. (w/v) of

ice-cold 0.01 M Tris buffer containing 0.1 % Triton X-100 (w/v), pH 7.0 . The supernatant was run for DBH assay as that described for brain homogenate, using different concentrations of cupric ion to inactivate the possible endogenous inhibitors. As shown in Table 4-5, a minute but definite amount of DBH was found in dog kidneys with intact sympathetic innervation. It appears that endogenous inhibitors are present only in very small amounts or absent in normal renal tissue; hence no exogenous copper is required for optimal DBH activity. Three weeks after severing the renal nerves, DBH activity in the denervated kidney decreased to a level not different from that of the blank. These findings again substantiate the fact that the DBH assay procedure is specific and only measures the DBH activity in a structure innervated by sympathetic nerves.

TABLE 4-5 Effect of Renal Denervation on DBH Activity in Dog Renal Cortex.

Renal denervation was carried out 3 weeks before sacrifice. Values are means ± S.E.M., expressed in nmoles oct./gm/hr, n = 4.

Cu^{++} Concentration (μM)	D B H Activity (nmoles oct./gm/hr)	
	Renal Nerve Intact	Renal Nerve Severed
0	3.8 ± 0.4	0
25	2.4 ± 0.4	0
50	2.1 ± 0.3	0

DISCUSSION With a minor modification of the assay procedure of Molinoff et al. (1971), I was able to measure the DBH activity in regions of rat brain. I confirmed the observation that optimal enzyme activity in brain

tissue can be obtained only after adding appropriate amounts of cupric ions to the reaction mixture. The action of exogenous copper is presumably on the neutralization of endogenous inhibitors of DBH. Too little copper will not adequately inactivate the inhibitors, whereas too much copper will inhibit both DBH and PNMT. In three brain regions studied, i.e. the hypothalamus, brain stem and hippocampus, the optimal copper concentration for DBH was always 75 μM. Different amounts of copper for different regions of the brain have been reported by others. For the hypothalamus, reported values were 110 μM (Coyle and Axelrod, 1972) or 130 μM (Reis and Molinoff, 1972), while for the brain stem they were 80 μM (Coyle and Axelrod, 1972) or 96 μM (Reis and Molinoff, 1972). My values for copper requirement for three brain regions are lower than theirs. This may indicate that in the process of homogenizing the brain and running DBH reaction, some of the DBH inhibitors were inactivated so that not as much copper was required for the optimal DBH activity.

The specificity of the reaction was tested by the chromatographic study which revealed that the main radioactive product of the reaction was exactly the one expected. When noradrenergic neurons were destroyed by chemical sympathectomy with 6-hydroxydopamine or by mechanical denervation, the DBH activity decreased markedly, again suggesting that the assay for DBH is specific and measures primarily the DBH in noradrenergic neurons.

The accuracy and reproducibility of the assay is illustrated by the small magnitude of intra- and interassay variation. Also the duplicate values for each sample were mostly close to each other. In view of the low counts in blank tubes, and the wide range of amounts of octopamine used in the plotting of the standard curve, the assay is sensitive and quantitative as well. Once the optimal reaction conditions have been determined, this assay is rapid, convenient and reproducible. In conclusion, the

coupled DBH assayed established by me appeared to be specific, accurate and sensitive, and was adequate for my study on the changes in DBH activity in brain regions after physiological manipulation.

==== C H A P T E R II - 5 ====

DISTRIBUTION OF DBH ACTIVITY IN REGIONS OF RAT BRAIN AND ITS DIURNAL FLUCTUATION

OBJECTIVE To study the regional distribution of DBH in rat brain and its diurnal fluctuation.

INTRODUCTION Before investigating changes of DBH in the hypothalamus, brain stem and hippocampus of the rat during endocrine manipulations, it is essential to determine the normal, resting levels of DBH activity in these regions. In addition, circadian variation in plasma adrenocortical steroids is well established (Critchlow, 1963) and a 24-hour rhythm in brain norepinephrine content has also been reported (Friedman and Walker, 1968; Manshardt and Wurtman, 1968; Asano, 1971). Therefore, determination of diurnal fluctuation of dopamine-β-hydroxylase in brain regions was an important preliminary step in my studies.

METHODS AND RESULTS

A. Regional Distribution of DBH in the Brain.

Male Sprague-Dawley rats, weighing 150-200 grams, were used in eight experiments carried out during a period of 24 months. Animals were caged in pairs for at least 7 days before sacrifice in a room at 22 ± 1°C with automatic light control that provided 12 hour light, 12 hour dark days. Food and water were given ad libitum. Control rats were decapitated immediately after removal from the cage without any manipulation. The hypothalamus, brain stem and hippocampus were dissected out and measured

for DBH activity according to the procedures described in Chapter II-4 (pp. 84-86).

Values of DBH activity in the three brain regions, determined in eight different experiments, are listed in Table 5-1. The highest activity of the enzyme was in the hypothalamus, ranging between 280 and 340 nmoles oct. per gram of tissue per hour, and most freguently between 300 and 320 units. The brain stem contained moderately high levels of DBH activity, ranging between 200 and 240 units with one exception, in which the DBH activity was only 177.5 units possibly due to the inappropriate assay, or less likely due to the variation of animals. There was relatively small amount of DBH in the hippocampus, only about one fifth of that in the hypothalamus.

TABLE 5-1 DBH Activity in Regions of Normal Rat Brain.

Values are means ± S.E.M., expressed in nmoles oct./gm/hr.
The number of animals in experiment #1 was 60, that in exp.#3 was 4, and that in all other experiments was 6.

Experiment Number	D B H Activity (nmoles/gm/hr)		
	Hypothalamus	Brain Stem	Hippocampus
1	344.6 ± 5.9	237.3 ± 6.8	
2	304.6 ± 11.9		
3	279.9 ± 2.6	177.5 ± 14.3	
4	338.9 ± 5.5	234.0 ± 10.4	74.3 ± 2.0
5	318.1 ± 7.0	221.0 ± 8.3	68.6 ± 4.0
6	312.4 ± 5.7	211.4 ± 4.2	66.1 ± 0.6
7	312.4 ± 13.3	221.7 ± 14.8	63.3 ± 4.6
8	301.6 ± 11.9	203.9 ± 5.8	61.3 ± 3.5

It is of interest to examine the distribution of DBH in different parts of the hypothalamus. The dissected block of the hypothalamus was further cut in the coronal plane into anterior and posterior halves, or cut in the plane parallel to the brain base into dorsal and ventral halves. The division was made with the naked eye. Care was taken to cut the hypothalamus into as equal halves as possible.

Table 5-2 shows the DBH activity in anterior and posterior halves of the rat hypothalamus. The anterior half of the hypothalamus contains more DBH than the posterior. Similarly, Table 5-3 shows that the ventral part of the hypothalamus has a higher amount of DBH than the dorsal part.

TABLE 5-2 DBH Activity in Anterior and Posterior Halves of Hypothalamus.

Part of Hypothalamus	Number of Animals	DBH Activity (nmoles/gm/hr) Mean ± S.E.M.
Anterior	6	337.1 ± 20.5
Posterior	6	215.3 ± 11.1

TABLE 5-3 DBH Activity in Ventral and Dorsal Halves of Hypothalamus.

Part of Hypothalamus	Number of Animals	DBH Activity (nmoles/gm/hr) Mean ± S.E.M.
Ventral	6	283.4 ± 7.3
Dorsal	6	233.8 ± 18.6

B. Diurnal Fluctuation of DBH Activity in Regions of Rat Brain.

Male Sprague-Dawley rats, weighing 200-220 grams, were caged in pairs for one week before sacrifice in a room at 22 ± 1°C with automatic light control that provided 14 hour light (0515 - 1915 Pacific Standard Time) and 10 hour dark each day. Food and water were given ad libitum. The animals were killed by decapitation immediately after removal from the cage at 0500, 0700, 0900, 1200, 1500, 1700, 1900, 2100, 2400 and 0300 hours. The hypothalamus and brain stem were dissected out and the DBH activity measured. As shown in Table 5-4, there is no marked fluctuation of DBH activity in either hypothalamus or brain stem: the difference in DBH activity between any two time points is not stastistically significant (student "t" test, $P > 0.05$). However, when brain stem DBH values for the afternoon (3 pm and 5 pm) are pooled to be compared with those for the night (9 pm and 12 midnight), the difference reaches the significant level (215.2 ± 13.9 vs 262.0 ± 18.1, $n = 12$, $P < 0.05$). Thus there is a slight tendency toward increased DBH activity during the light-off period when the rats are most active.

Because of the change in animal room when the subsequent studies were undertaken, the lighting schedule was set at 12:12 light-dark period, rather than the 14:10 light-dark of this particular study.

DISCUSSION The present study comfirms and extends the observation by others of the presence of DBH in the brain (Udenfriend and Creveling, 1959; Bonnay et al., 1966; Coyle and Axelrod, 1972; Reis and Molinoff, 1972). Like the levels of norepinephrine (Glowinski and Iversen, 1966), the activity of the enzyme varies in different regions of the brain. In general, the relative regional levels of amine and DBH parallel each other. Of the

TABLE 5-4 DBH Activity in Regions of Rat Brain: Circadian Fluctuation.

The light in the animal room was on at 5:15 am and off at 7:15 pm.
Values are means ± S.E.M. of 6 rats, expressed in nmoles/gm/hr.

TIME OF THE DAY	5 am (LIGHT-ON at 5:15 am)	7 am	9 am	12 Nn	3 pm	5 pm	7 pm (LIGHT-OFF at 7:15 pm)	9 pm	12 Nt	3 am	5 am
HYPOTHALAMUS											
Mean	326.6	344.7	352.7	343.2	346.6	341.7	332.5	358.4	353.0	346.3	326.6
S.E.M.	±18.4	±19.6	±28.5	±10.8	±26.3	±18.7	±16.5	±22.4	±18.3	±14.3	±18.4
BRAIN STEM											
Mean	246.2	257.3	226.0	219.8	218.2	212.2	243.1	268.3	255.6	226.2	246.2
S.E.M.	±14.1	±16.9	±22.1	±32.1	±23.9	±16.4	±19.7	±27.3	±25.9	±11.3	±14.1

215.2 ± 13.9 (n =12) — 262.0 ± 18.1 (n =12)

$P < 0.05$

three areas examined, the highest DBH activity is in the hypothalamus, followed by the brain stem; these are areas of high norepinephrine content in the brain. The hippocampus contains low levels of DBH and norepinephrine. The absolute values of DBH in the hypothalamus and brain stem are higher than those reported by Coyle and Axelrod (1972) and by Reis and Molinoff (1972). The reasons for this discrepancy are not clear. It may be due to modifications of the assay procedures: there was less cupric ion in the reaction mixture, and the DBH reaction was run in a more acidic medium since it has been shown that the optimal pH for DBH is 5.5 , rather than 6.0, when tyramine is used as the substrate (Creveling et al., 1962; Wooten et al., 1973).

It has been shown that the anterior hypothalamus contains higher levels of norepinephrine than the posterior hypothalamus (Stefano et al., 1965; Manshardt and Wurtman, 1968; Palkovits et al., 1974). The ventral half of the hypothalamus has also been shown to have a higher content of norepinephrine than the dorsal half (Cuello et al., 1973; Palkovits et al., 1974). My observation that the anterior and ventral hypothalamus contained higher DBH activity than their counterparts is therefore consistent with the findings for norepinephrine content.

Circadian variations of norepinephrine content in the rat brain have been studied (Asano, 1971). Norepinephrine showed low values during the light period and a peak in the dark. Friedman and Walker (1968) found that midbrain norepinephrine content of the rat was higher during the dark phase of the light cycle. Significant daily rhythms of norepinephrine levels have also been observed in the anterior and the posterior hypothalamus of the rat (Manshardt and Wurtman, 1968). The norepinephrine content of both regions was greatest at the middle of the dark period; in the anterior hypothalamus, the norepinephrine content was low throughout

the light period, while in the posterior hypothalamus, the nadir was reached at the end of the dark period. However, the magnitude of the fluctuation was not marked (about 10 %). Therefore, the absence of a significant diurnal variation of DBH activity in the hypothalamus and the brain stem is not surprising. The change in the functional states of noradrenergic neurons in the rat brain during light and dark cycles is not accompanied by a parallel change in DBH activity. Nevertheless, it is worth noting that there is a slight tendency toward the increased DBH activity in the brain stem of rats with increased motor activity after they have been in the darkness for a period of time. Since there is no marked fluctuation of DBH activity during the day time, all of the subsequent experiments on the change in DBH activity were scheduled to be carried out between 10 am and 2 pm without sticking to a particular time point in the day.

==== C H A P T E R II - 6 ====

EFFECT OF ANESTHETICS AND STRESS ON BRAIN DBH ACTIVITY

OBJECTIVE To study the effect of anesthetic agents (pentobarbital or ether) and stress (subcutaneous injection procedure, surgical laparotomy or immobilization) on the DBH activity in rat brain.

INTRODUCTION General anesthesia is required in most animal experiments and human surgery. In the subsequent experiments, the removal of rat adrenal glands required laparotomy under general anesthesia. Hence, it was rational to examine the effect of widely used general anesthetic agents, pentobarbital and ether, on brain DBH activity. Many stressful stimuli that increase ACTH-corticosteroid secretion are also associated with changes in the functional activity of central noradrenergic neurons (see Chapter I-9). In essence, stress appears to decrease steady-state levels and increase the dynamic turnover of norepinephrine in various regions of the brain. Dopamine-β-hydroxylase activity in the brain after stress has not been studied. The following studies were carried out to determine DBH activity in regions of rat brain after different stressful procedures, such as the presumably painful stimulation of subcutaneous injection, surgical laparotomy, and immobilization. It should be noted that ether inhalation in the rat increases the secretion of ACTH and corticosteroids, and hence is a stressor (Dallman and Yates, 1968).

METHODS AND RESULTS

Male Sprague-Dawley rats, weighing 160-180 grams, were used for the studies. Pentosol (sodium pentobarbital, 65 mg/ml) was the product of Burns-Biotec Laboratories Inc., Oakland, California. Ether for Anesthesia was purchased from Mallinckrodt Chemical Works, St. Louis.

A. Effect of Pentobarbital on Hypothalamic DBH Activity.

Experimental rats received 50 mg/kg of sodium pentobarbital by the intraperitoneal route, and were killed at various time intervals after the injection. Control rats did not receive any injection. After decapitation, the brain was quickly removed and placed on dry ice. The hypothalamus was dissected out in frozen state and DBH activity assayed according to the procedures already described. As shown in Table 6-1, there is no change in DBH activity after pentobarbital injection for the time up to 120 minutes. The differences between experimental animals and controls are not significant ($P > 0.05$).

TABLE 6-1 Effect of Pentobarbital Anesthesia (50 mg/kg, i.p.) on Hypothalamic DBH Activity. Values are means ± S.E.M.

Time after Injection (minutes)	Number of Animals	Hypothalamic DBH Activity (nmoles oct./gm/hr)
Control*	6	304.6 ± 11.9
5	6	297.9 ± 19.9
10	6	322.0 ± 19.9
20	6	275.2 ± 9.6
30	6	303.6 ± 13.8
40	6	307.9 ± 9.9
60	6	283.5 ± 20.2
90	5	291.9 ± 20.0
120	5	304.5 ± 11.1

* Control rat did not receive any injection.

B. Effect of Ether on DBH Activity in Regions of Rat Brain.

Table 6-2 represents the results of three separate experiments. Fifteen minutes and 2 hours after rats were exposed to ether vapor for 2-3 minutes, there was no change in DBH activity in the anterior hypothalamus, posterior hypothalamus or brain stem, at a time when the pituitary-adrenocortical system has been activated. However, when the rats were sacrificed 24 hours after ether exposure, DBH activity in the hypothalamus increased by 10-30 %. Changes in brain stem DBH activity were not consistent: one experiment showed an increase while the other showed no change.

C. Effect of Subcutaneous Injection on Brain DBH Activity.

Injection stress consisted of holding the rat firmly and inserting a 20 x 1" needle into the subcutaneous space over the back to inject 0.2 to 0.5 ml of peanut oil. The injection was made either once at 4 hours before the rat was killed, or once daily for 5 days prior to sacrifice. Table 6-3 shows that in neither instance was there any change in DBH activity in the hypothalamus, brain stem or hippocampus after holding the rat and performing a presumably painful subcutaneous injection.

D. Effect of Surgical Stress on DBH Activity in Rat Brain.

Under pentobarbital anesthesia, a 1.5-inch skin incision was made over the midline of the back, and the abdominal cavity was opened by making two small holes, one on each side of the back. The incision was then closed by wound clips. Control rats did not receive any anesthesia or surgery. As shown in Table 6-4, there was no significant change ($P's > 0.05$) in DBH activity in the hypothalamus, brain stem or hippocampus, either 24 hours or 5 days after the surgery.

TABLE 6-2 Effect of Ether Inhalation on DBH Activity in Rat Brain.

Values are means ± S.E.M., expressed in nmoles/gm/hr.
Numbers in parentheses are numbers of the rats in each group.

Experiment 1. Rats were put in an ether jar for 2 minutes and killed 15 minutes after ether exposure. Control rats did not receive any manipulation.

	Anterior Hypothalamus	Posterior Hypothalamus	Brain Stem
Control (6)	337.1 ± 20.5	215.3 ± 11.1	176.6 ± 8.5
Ether 15 min (6)	357.3 ± 27.4	198.3 ± 12.6	175.2 ± 16.4

Experiment 2. Rats were put in an ether jar for 3 minutes and killed 2 hours or 24 hours after ether exposure. Control rats did not receive any manipulation.

	Anterior Hypothalamus	Posterior Hypothalamus	Brain Stem
Control (6)	289.4 ± 8.0	138.0 ± 11.3	76.8 ± 5.1
Ether 2 hrs (6)	302.9 ± 10.6	141.4 ± 8.5	88.2 ± 8.9
Ether 24 hrs (5)	376.0 ± 26.9*	174.6 ± 18.4	113.8 ± 10.6*

* $P < 0.01$ vs controls.

Experiment 3. Rats were put in an ether jar for 3 minutes and killed 12 hours or 24 hours after ether exposure. There was no measurement of DBH in the rats without ether exposure. Comparison is made between 12 hours vs 24 hours of ether exposure.

	Hypothalamus	Brain Stem
Ether 12 hrs (6)	277.5 ± 5.0	264.0 ± 5.8
Ether 24 hrs (6)	308.3 ± 4.3**	248.5 ± 7.0

** $P < 0.001$ vs Ether 12 hours.

TABLE 6-3 Effect of Injection Stress on DBH Activity in Rat Brain.

Values are means ± S.E.M., expressed in nmoles/gm/hr. Numbers in parentheses are numbers of the rats in each group. Control rats did not receive any manipulation.

Experiment 1. Injected rats received 0.2-0.5 ml of peanut oil s.c. and were killed 4 hours after injection. There are three studies in this experimental group.

	Hypothalamus	Brain Stem	Hippocampus
a. Control (3)	314.0 ± 25.7	236.7 ± 18.8	
Injected (3)	341.2 ± 2.3	241.4 ± 2.9	
b. Control (6)	338.9 ± 5.5	234.0 ± 10.4	74.3 ± 2.0
Injected (6)	350.0 ± 4.1	233.5 ± 6.8	69.2 ± 3.2
c. Control (6)	318.1 ± 7.0	221.0 ± 8.3	68.6 ± 4.0
Injected (6)	318.3 ± 6.0	222.4 ± 12.2	65.6 ± 4.9

Experiment 2. Injected rats received 0.5 ml of peanut oil s.c. daily for 5 days and were killed 24 hours after the last injection.

Control (6)	301.6 ± 11.9	203.9 ± 5.8	61.3 ± 3.5
Injected (7)	314.0 ± 8.2	201.1 ± 6.5	64.7 ± 2.5

TABLE 6-4 Effect of Surgical Stress on DBH Activity in Rat Brain.

Values are means ± S.E.M., expressed in nmoles/gm/hr. Numbers in parentheses are numbers of the rats in each group. Surgical stress consisted of pentobarbital anesthesia and sham adrenalectomy. Control rats did not receive manipulation.

Time after Surgery	Hypothalamus	Brain Stem	Hippocampus
a. 24 hours			
Control (6)	312.4 ± 5.7	211.4 ± 4.2	66.1 ± 0.6
Operated (6)	309.7 ± 4.0	224.3 ± 4.7	63.7 ± 1.2
b. 5 days			
Control (6)	312.4 ± 13.3	221.7 ± 14.8	63.3 ± 4.6
Operated (7)	320.7 ± 7.9	248.2 ± 11.5	67.2 ± 3.0

E. Effect of Immobilization on DBH Activity of Rat Brain.

For acute immobilization, rat were kept restrained for 10 minutes in aluminum cans with holes to ensure adequate heat dissipation, and were killed 30 minutes after release from the restraint. For repeated immobilization, rats were restrained in the same kind of cans 2 hours in the morning and 2 hours in the afternoon each day for 10 days. On eleventh day the rats again received 10 minutes of immobilization and were then killed 30 minutes after release. Control rats did not receive any manipulation. The experiment was scheduled so that all the rats were killed on the same day. DBH activity in the anterior hypothalamus, posterior hypothalamus and brain stem was determined. As shown in Table 6-5, there was a specific change in brain DBH activity after restraint stress. Shortly after the rat was immobilized, DBH activity in the anterior hypothalamus decreased by about 20 %. This decrease in DBH activity was curtailed if the rat had been repeatedly restrained during the previous 10 days. There was no change in DBH activity in the posterior hypothalamus and brain stem after either acute or repeated immobilization.

TABLE 6-5 Effect of Immobilization on DBH Activity in Rat Brain.

Values are means ± S.E.M., expressed in nmoles/gm/hr.
There were 6 rats in each group.
Control rats did not receive any manipulation.

	Anterior Hypothalamus	Posterior Hypothalamus	Brain Stem
Control	302.1 ± 7.0	163.4 ± 11.1	103.4 ± 8.3
Acute Restraint	225.3 ± 8.1*	141.7 ± 4.8	102.7 ± 8.6
Repeated Restraint	271.5 ± 8.8	145.2 ± 7.9	103.5 ± 11.1

* $P < 0.003$ vs control or repeated restraint.

DISCUSSION Phenobarbital has been reported to prevent the electric shock-induced depletion of norepinephrine in the brain stem of the rat (Maynert and Levi, 1964). Although the detailed mechanism is not known, it seems likely that phenobarbital acts by blocking nervous connections to the adrenergic neurons in the brain. That sedation per se is not responsible for the inhibitory effect of this drug on the release of norepinephrine was proved by the failure of morphrine to prevent depletion (Maynert and Levi, 1964). Subsequent studies showed that during barbiturate anesthesia the turnover of noradrenergic neurons in rat brain was diminished (Corrodi et al., 1971; Persson and Waldeck, 1971). The present study indicate that there is no significant change in DBH content after pentobarbital injection; this suggests that the decreased turnover of norepinephrine in the brain after barbiturate anesthesia is not a consequence of DBH deficiency.

Ether inhalation in the rat activates the pituitary-adrenocortical system (Dallman and Yates, 1968; Carr and Moore, 1968). Shortly after rats were exposed to ether vapor, there was no change in DBH activity in the hypothalamus and brain stem, this is consistent with the finding of lack of acute change in brain norepinephrine and dopamine content after ether exposure (Carr and Moore, 1968). However, DBH activity in the hypothalamus had increased by 10-30 % when the rats were sacrificed 24 hours after ether anesthesia. This increase in DBH activity is probably not attributable to the direct action of ether on the enzyme molecule, since no stimulation could be observed shortly after the exposure. Rather, it might be due to the increase in impulse flow in noradrenergic neurons, a direct effect of ether on the neuronal activity or an effect secondary to the elevated pituitary-adrenocortical secretion after ether anesthesia.

Injection stress and surgical stress have been reported to provoke a transient but definite increase in corticosterone secretion in the rat (Dallman and Jones, 1973). I failed to find any change in DBH activity in regions of rat brain 4 hours after injection stress or after repeated daily injection stress for 5 days. Similarly, no change in DBH activity could be obtained either 24 hours or 5 days after surgical stress. These findings indicate that DBH in the brain is not sensitive to those stressful procedures.

Shortly after the rat was subjected to immobilization stress, DBH activity in the anterior hypothalamus decreased by about 20 % without concomitant decrease in the posterior hypothalamus or brain stem. The decrease in DBH activity in the anterior hypothalamus may result from an acute discharge of DBH out of the neuronal endings without an immediate resupply from the cell body. In view of the increased norepinephrine turnover after restraint stress with no change or a decrease in norepinephrine content (Table I-4, p. 54), it is suggested that there is also an acute discharge of neurotransmitter from the nerve endings. Lastly, the restoration of DBH activity after repeated immobilization for 5 days may represent the phenomenon of habituation or may be a result of increased DBH manufacture after chronic stress.

==== C H A P T E R II - 7 ====

EFFECT OF CORTICOSTERONE TREATMENT ON BRAIN DBH ACTIVITY

OBJECTIVE To study the effect of corticosterone excess on DBH activity in the hypothalamus, brain stem and hippocampus of the rat.

INTRODUCTION One way to study the effect of a given kind of hormone is to administer it in pure form to an animal with or without the endocrine glands that secrete the hormone named. Consequently, I treated intact rats with large dose of corticosterone, the principal secretory product of rat adrenal cortex, to explore the effect of this glucocorticoid on DBH activity in rat brain.

METHODS AND RESULTS

Male Sprague-Dawley rats, weighing 150-170 grams, were housed in pairs in the animal room with a lighting schedule of 12 hours light and 12 hours dark. Rat chow and water were given ad libitum. Corticosterone (Sigma, St. Louis) was suspended in peanut oil for subcutaneous injection. Some rats received peanut oil to serve as the vehicle-injected controls. Another group of control animals did not receive any injection. The rats were sacrificed by decapitation. Trunk blood was collected in tubes containing sodium heparin (Lipo-Hepin, Riker Laboratories, Inc., Northridge, California). The blood was centrifuged and the plasma used for determination of corticosterone by a competitive protein-binding radioassay (Murphy, 1967). The hypothalamus, brain stem and hippocampus were dissected

out for the measurement of DBH activity by the method described earlier.

In order to achieve sustained high levels of corticosterone in rat plasma, rats were given a large dose of corticosterone (10 mg/100 gm B.W.). After subcutaneous injection total plasma corticosterone concentration (free and Protein-bound) reached a plateau of 120-150 μg/100 ml within 30 minutes and remained at that high level for at least 4 hours (Fig. 7-1). Twenty-four hours later plasma corticosterone was still at a level comparable to that attained after stress (47.1 ± 2.2 μg %). The normal plasma level of corticosterone in the rat is less than 10 μg % (Dallman and Jones, 1973).

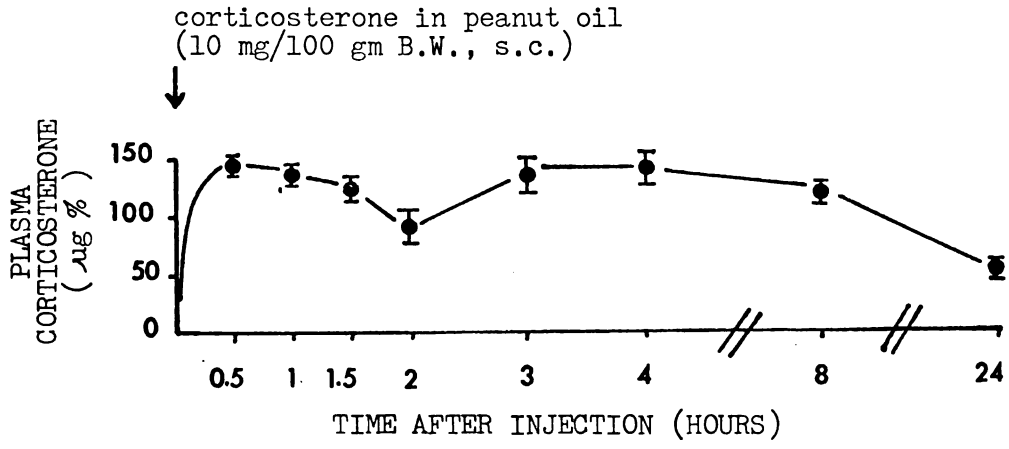


Fig. 7-1 Plasma Corticosterone Concentration at Various Time after Injection of Corticosterone.

Four hours after the large dose of corticosterone, DBH activity in the hypothalamus increased by 7 % ($P < 0.02$), whereas that of brain stem and hippocampus remained unchanged (Table 7-1). When the rat received this treatment daily for 5 days, DBH activity in all three regions of the brain increased by 15-20 % (Table 7-2).

TABLE 7-1 DBH Activity Measured 4 hours after a Large Dose of Corticosterone (10 mg/100 gm B.W.).

Values are means ± S.E.M., expressed in nmoles/gm/hr. Numbers in parentheses are numbers of rats. Control rats did not receive any injection.

INJECTION	HYPOTHALAMUS	BRAIN STEM	HIPPOCAMPUS
Control (6)	318.1 ± 7.0	221.0 ± 8.3	68.6 ± 4.0
Peanut oil (6)	318.3 ± 6.0	222.4 ± 12.2	65.6 ± 4.9
Corticosterone (6)	340.9 ± 3.4*	221.4 ± 7.8	64.8 ± 4.7

* $P < 0.02$ vs normal control or oil-injected .

TABLE 7-2 DBH Activity after Daily Injection of Large Doses of Corticosterone (10 mg/100 gm B.W./day) for 5 Days.

Values are means ± S.E.M., expressed in nmoles/gm/hr. Numbers in parentheses are numbers of rats. The daily injection was made into the subcutaneous tissue of the back for 5 days. Rats were killed 24 hours after the last injection. Control rats received no injection.

INJECTION	HYPOTHALAMUS	BRAIN STEM	HIPPOCAMPUS
Control (6)	301.6 ± 11.9	203.9 ± 5.8	61.3 ± 3.5
Peanut oil (7)	314.0 ± 8.2	201.1 ± 6.5	64.7 ± 2.5
Corticosterone (7)	346.0 ± 10.4*	248.5 ± 7.6*	75.3 ± 2.5*

* $P < 0.03$ vs normal control or oil-injected.

DISCUSSION Assuming 85 % of corticosterone is bound to plasma protein when total corticosterone in the plasma is 150 µg % (calculated from Fig. 3 of Fortier et al., 1970), the concentration of free corticosterone in the extracellular fluid of the rat receiving 10 mg/100 gm of the steroid

is about 0.6 μM. Four hours after the brain cells have been bathed in such kind of tissue fluid, DBH activity in the hypothalamus increased by 7 %, but not in other regions of the brain. Keen and McLean (1974) demonstrated that dexamethasone increased the DBH content by 80 % after 6 hours of incubation of isolated rat superior cervical ganglia. However, the concentration of dexamethasone in the incubation medium was very high (100 μM). After repeated daily injection of corticosterone for 5 days, I found a significant increase in the DBH activity in the hypothalamus, brain stem and hippocampus.

The exact mechanism that leads to the increase in DBH activity is not known. A direct activation of DBH molecules by the steroid within 4 hours is not likely, since the increase in DBH activity could only be detected in the hypothalamus, not in the brain stem or hippocampus. However, it is possible that the increase of DBH in the hypothalamus is a consequence of increased supply from cell bodies in the brain stem, where a normal level of DBH is maintained by a concomitant increase in protein synthesis.

Prolonged influence by corticosterone for 5 days leads to an increase in DBH activity in noradrenergic endings (hypothalamus and hippocampus) and cell bodies (brain stem). This may be a manifestation of hormone induction which results in the increased manufacture of enzyme molecules. Similar mechanism has been proposed for the induction of DBH in the sympathetic ganglia *in vitro* (Keen and McLean, 1974) and for the induction of tryptophan hydroxylase in the midbrain of the rat *in vivo* (Azmitia and McEwen, 1969).

McEwen et al. (1969) have shown that radioactive corticosterone is retained and concentrated by a limited-capacity uptake process in the hippocampus and septum of adrenalectomized rats, indicating that the limbic

system of the rat brain is differentiated with respect to hormone uptake. Subsequent autoradiographic study demonstrated that the nuclei of neurons in the hippocampus were heavily labeled but radioactivity was relatively sparse in the cytoplasm and axons (Gerlach and McEwen, 1972). My finding that no change in hippocampal DBH activity 4 hours after a single injection of corticosterone, in contrast to that in the hypothalamus, suggests that the noradrenergic structures in the hippocampus are relatively insensitive to the effect of corticosteroids.

==== C H A P T E R II - 8 ====

EFFECT OF ADRENALECTOMY WITH OR WITHOUT CORTICOSTERONE REPLACEMENT ON DBH ACTIVITY IN RAT BRAIN

OBJECTIVE (1) To study the effect of removal of adrenal glands, with or without corticosterone replacement, on DBH activity in the hypothalammus, brain stem and hippocampus of the rat.
(2) To study the effect of interrupting sensory input from one adrenal gland on DBH activity in individual sides of the hypothalamus and brain stem.
(3) To study the effect of ovariectomy on brain DBH activity.

INTRODUCTION The most common method employed to study the effect of a given kind of hormone is to remove the glands that secrete the hormone. The change in DBH activity in various regions of the rat brain was studied after bilateral adrenalectomy, with or without corticosterone replacement. In addition, there may be a sensory input from adrenal glands to the central nervous system that ultimately influences the morphology and function of the hypothalamus (pp. 312-315 in Szentágothai et al., 1962). The change of DBH activity in individual sides of the hypothalamus and brain stem was therefore studied after unilateral adrenalectomy or simply interrupting the sensory input from the adrenal gland on one side by squeezing the adrenal pedicle. In a preliminary experiment, DBH activity in the brain was also measured after ovariectomy with or without estrogen replacement.

METHODS AND RESULTS

A. Effect of Bilateral Adrenalectomy on Brain DBH Activity.

Male Sprague-Dawley rats, weighing 150-170 grams, were housed in an animal room with automatic control in lighting schedule (12-hour light and 12-hour dark). Under pentobarbital anesthesia a 1.5-inch skin incision was made over the midline of the back, and the abdominal cavity was opened by making two small holes, one on each side of the back. Both adrenal glands were removed and the incision wound was closed with wound clips. In sham-operated rats, adrenal glands were looked at, but not touched. Control rats did not receive anesthesia or operation. All rats were given standard laboratory chow ad libitum. Sham-operated or control rats drank tap water, while adrenalectomized rats were maintained on 0.9 % NaCl solution. Rats were killed by decapitation at various time intervals after the operation. The trunk blood was collected in tubes containing sodium heparin and total plasma corticosterone was measured (Murphy, 1967). DBH activity in the hypothalamus, brain stem and hippocampus was determined by the method described in Chapter II-4.

Table 8-1 summarizes the results of three experiments. The completeness of adrenalectomy is indicated by the low plasma corticosterone levels that are below the sensitivity of the assay. Sham-operated rats did not have altered DBH activity in any region of the brain. Twenty-four hours after bilateral adrenalectomy, DBH activity in the hypothalamus and brain stem decreased by 10 %, but DBH activity in the hippocampus did not show any change. When rats were sacrificed 48 hours after adrenalectomy, DBH activity in the hypothalamus and brain stem decreased to 86 % and 78 %, respectively, of that of sham-operated controls. Five days after adrenalectomy DBH activity in the hypothalamus had returned to normal levels, whereas that in the brain stem remained low.

TABLE 8-1 DBH Activity in Rat Brain after Bilateral Adrenalectomy (AdX).

Values are means ± S.E.M.; DBH activity is expressed as nmoles oct./gm/hr, and total plasma corticosterone (B) is expressed as μg/100 ml. Numbers in parentheses are numbers of the rats. Control rats received no manipulation.

	D B H ACTIVITY (nmoles/gm/hr)			PLASMA B
OPERATION	HYPOTHALAMUS	BRAIN STEM	HIPPOCAMPUS	(μg %)
Exp. 1. Rats were killed 24 hours after the operation.				
Control (6)	312.4 ± 5.7	211.4 ± 4.2	66.1 ± 0.6	5.5 ± 1.9
Sham-op (6)	309.7 ± 4.0	224.3 ± 4.7	63.7 ± 1.2	11.0 ± 2.1
Ad X (6)	281.8 ± 6.7*	184.9 ± 9.2*	63.1 ± 3.4	< 1.5
Exp. 2. Rats were killed 2 days after the operation.				
Sham-op (6)	321.4 ± 8.7	230.4 ± 8.6		9.3 ± 3.0
Ad X (10)	275.1 ± 7.1@	178.8 ± 5.5@		1.1 ± 0.1
Exp. 3. Rats were killed 5 days after the operation.				
Control (6)	312.4 ± 13.3	221.7 ± 14.8	63.3 ± 4.6	3.4 ± 0.4
Sham-op (7)	320.7 ± 7.9	248.2 ± 11.5	67.2 ± 3.0	10.9 ± 2.8
Ad X (7)	310.3 ± 8.7	188.2 ± 8.7#	63.3 ± 1.6	< 1.5

* $P < 0.01$ vs sham-operated or unoperated control.

@ $P < 0.01$ vs sham-operated.

\# $P < 0.001$ vs sham-operated; $P < 0.07$ vs unoperated control.

B. Effect of Corticosterone Replacement in Adrenalectomized Rats on DBH Activity in Regions of the Brain.

Rats were adrenalectomized or sham-operated under pentobarbital anesthesia two days before sacrifice. All the adrenalectomized rats were

maintained on normal saline. Corticosterone suspension or vehicle (peanut oil) was injected subcutaneously either 4 hours or 24 hours before sacrifice. The results of two experiments are summarized in Table 8-2.

TABLE 8-2 Effect of Corticosterone Replacement in Adrenalectomized Rats on Brain DBH Activity.

Values are means ± S.E.M.; DBH activity is expressed as nmoles oct./gm/hr, and plasma corticosterone (B) is expressed as μg/100 ml. Numbers in parentheses are numbers of the rats.
Rats were adrenalectomized (AdX) or sham operated under pentobarbital anesthesia 2 days before sacrifice.
AdX rats were maintained on normal saline. Corticosterone or peanut oil was injected s.c. to AdX rats 4 or 24 hours before sacrifice.

OPERATION AND INJECTION	DBH ACTIVITY (nm/gm/hr) HYPOTHALAMUS	DBH ACTIVITY (nm/gm/hr) BRAIN STEM	PLASMA B (μg %)
Exp. 1 Injection 4 hours before sacrifice.			
Sham, without inj. (6)	321.4 ± 8.7	230.4 ± 3.7	9.3 ± 3.0
AdX, oil inj. (5)	272.8 ± 6.4	179.7 ± 2.9	1.1 ± 0.1
AdX, 5 mg B/kg (5)	274.4 ± 10.4	179.1 ± 4.4	22.1 ± 4.9
AdX, 10 mg B/kg (5)	267.0 ± 10.4	182.6 ± 6.2	51.0 ± 2.9
AdX, 20 mg B/kg (5)	283.1 ± 7.1	180.4 ± 7.6	> 150
Exp. 2 Injection 24 hours before sacrifice.			
Sham, without inj. (6)	321.4 ± 8.7	230.4 ± 8.6	9.3 ± 3.0
AdX, oil inj. (5)	277.6 ± 7.6	178.2 ± 8.1	1.1 ± 0.1
AdX, 5 mg B/kg (5)	268.0 ± 9.1	180.8 ± 7.5	1.2 ± 0.1
AdX, 10 mg B/kg (6)	280.4 ± 9.2	177.7 ± 6.3	1.3 ± 0.2
AdX, 20 mg B/kg (6)	273.8 ± 9.0	181.7 ± 3.0	2.5 ± 0.5

As shown in Table 8-2, DBH activity in the hypothalamus and brain stem decreased by 15-25 % two days after adrenalectomy. A single dose of corticosterone, 5, 10 or 20 mg/kg B.W., 4 hours or 24 hours before sacrifice, did not reverse the decrease in DBH activity in the hypothalamus and brain stem, although plasma corticosterone levels were elevated for at least 4 hours after hormone replacement.

C. Effect of Unilateral Adrenalectomy on DBH Activity in the Two Sides of the Hypothalamus and Brain Stem.

After bilateral adrenalectomy, the hypothetical sensory input from adrenal glands into the central nervous system are absent. In order to explore the effect of neural input on DBH activity in the brain, the effect of unilateral adrenalectomy or adrenal denervation on DBH activity of the hypothalamus and brain stem was studied. To detect unilateral effects, the brain tissues were further divided in the midline and two halves studied separately.

Table 8-3 summarizes the results of two experiments. In the first one carried out in collaboration with Dr. M.F. Dallman, the operation was performed under light ether anesthesia 24 hours before sacrifice. Only the left side of the abdomen was opened. In the control group, the left adrenal gland was looked at, but not touched. In the denervated group, the left adrenal pedicle was squeezed by forceps to sever the adrenal nerves, but the gland was left in place. In the adrenalectomized group, the left adrenal gland was removed. Twenty-four hours after the operation, rats were decapitated and the hypothalamus and brain stem were dissected. DBH activity in right and left halves of the brain regions was measured separately. DBH values in right and left halves were compared by the "paired t" test (p. 58 in Ipsen and Feigl, 1970) to obtain P values.

TABLE 8-3 Effect of Unilateral Adrenalectomy or Adrenal Denervation on DBH Activity in Two Sides of Rat Brain Regions.

DBH values are means ± S.E.M., expressed in nmoles/gm/hr. Those in right and left halves of brain regions were compared by the "paired t" test and P's were obtained for each test. In all cases the P's were always greater than 0.20, indicating that the differences between two sides were not stastistically significant at 5 % level.

Exp. 1 The operation was performed under ether anesthesia 24 hours before sacrifice. Only the left side of the abdomen was opened. In the control group, the left adrenal gland was looked at, but not touched. In the denervated group (Denerv.), the left adrenal pedicle was squeezed, but the gland was left in place. In the adrenalectomized group (AdX), the left adrenal gland was removed. There were 6 rats in each group.

	HYPOTHALAMUS		BRAIN STEM	
OPERATION	Right Side	Left Side	Right Side	Left Side
Control	310.9 ± 5.3	305.6 ± 5.5	252.8 ± 8.5	244.1 ± 6.8
Denerv.	295.2 ± 8.0	299.5 ± 8.5	231.6 ± 7.1	228.4 ± 9.5
Ad X	310.9 ± 5.4	312.6 ± 4.5	243.8 ± 7.8	240.5 ± 9.2

Exp. 2 The operation was performed under pentobarbital anesthesia 2 days before sacrifice. Both sides of the abdomen were opened. The right adrenal gland was removed, whereas the left was only looked at. There were 8 rats in this experiment.

HYPOTHALAMUS		BRAIN STEM	
Right Side	Left Side	Right Side	Left Side
282.8 ± 2.8	283.8 ± 3.3	220.1 ± 4.0	227.3 ± 5.8

It is obvious that 24 hours after unilateral adrenalectomy or adrenal denervation on the left side, there was no difference in DBH activity in two sides of the hypothalamus and brain stem.

In the second experiment, the operation was carried under pentobarbital anesthesia two days before sacrifice. Both sides of the abdomen were opened. The right adrenal gland was removed, while the left was only looked at. DBH values in the right and left halves of the hypothalamus and brain stem were determined separately and were then compared by the "paired t" test. Again, the large P values for the two tests ($P's > 0.30$) indicated that there was no difference in DBH activity between the two sides of the hypothalamus and the brain stem 2 days after interruption of neural input from right adrenal gland.

D. Effect of Ovariectomy and Estrogen Replacement on DBH Activity in Regions of Rat Brain.

Bilateral ovariectomy was performed on 10-day-old female Sprague-Dawley rats, and they were killed two and half months later. Some rats were treated with estradiol benzoate in oil (Progynon, Schering Corporation, Bloomfield, New Jersey), 200 μg/rat/day, for 3 days before sacrifice. Control rats did not receive any manipulation.

As shown in Table 8-4, removal of ovaries led to atrophy of the uterus, whereas replacement of estrogen for 3 days markedly increased uterine weight. In contrast, the DBH levels in the hypothalamus and brain stem were not sensitive to the alteration in the concentration of ovarian hormones in the circulation.

DISCUSSION DBH activity in the hypothalamus and brain stem decreased by 10-25 % one and two days after bilateral adrenalectomy. While DBH activity in the brain stem remained low after 5 days, that in the hypothalamus returned back to normal. This latter finding is consistent with

TABLE 8-4 Effect of Ovariectomy and Estrogen Treatment on DBH Activity in Rat Brain.

DBH values are expressed in nmoles oct./gm/hr, means ± S.E.M.
Numbers in parentheses are numbers of the rats.
Bilateral ovariectomy (OvX) was performed 2.5 months before sacrifice. Some rats were treated with estradiol (E_2), 200 μg per rat per day for 3 days before sacrifice.
Control rats did not receive surgery or injection.

OPERATION	INJECTION		UTERUS WEIGHT (mg)	D B H ACTIVITY (nm/gm/hr) HYPOTHALAMUS	BRAIN STEM
Control	-	(5)	468.4 ± 53.0	292.7 ± 4.4	144.9 ± 7.9
OvX	-	(8)	36.9 ± 2.2	292.1 ± 9.8	158.2 ± 13.3
OvX	E_2	(8)	125.5 ± 9.7	287.0 ± 5.0	166.7 ± 6.9

the data reported by Kizer et al. (1974). No change in DBH activity in the hippocampus could be detected one day or five days after adrenalectomy.

To determine whether the absence of glucocorticoids was responsible for the decrease in DBH activity, corticosterone was replaced by injection in the adrenalectomized rats. Although 10 mg/100 gm was able to increase hypothalamic DBH activity by 7 % after 4 hours in rats with intact adrenals, 0.5, 1.0 or 2.0 mg/100 gm, given at 4 hours or 24 hours previously, failed to restore the enzyme activity in adrenalectomized rats. These doses of corticosterone produce plasma levels in the physiological range. Failure of corticosterone to restore the decreased DBH activity in adrenalectomized rats suggests that mere absence of glucocorticoids is not responsible for that decrease.

It is obvious that many other factors are missing in adrenalectomized

rats. In addition to glucocorticoids, mineralocorticoids (deoxycorticosterone and aldosterone) and catecholamines (epinephrine and norepinephrine) are normally secreted by the adrenal glands. It is possible that the deficiency in mineralocorticoids in adrenalectomized rats leads to the decrease in DBH activity. However, taking into account the maintenance of rats by normal saline and a partial salt-retaining effect of corticosterone, this explanation seems unlikely. The effect of adrenal catecholamines on DBH activity is also unknown from my study.

Szentágothai et al. (1962) has proposed that there is a neural input from each adrenal that reaches the contralateral side of the central nervous system. The receptors in the adrenal cortex or capsule may sense tissue pressure or some other parameter of the functional state of the adrenal cortex. In Szentagothai's experiments, the size of the cell nuclei in the ventromedial nucleus of the hypothalamus was enlarged if the adrenals were removed. The interruption of the nerves connecting the adrenal with the spinal cord induced the same change in nuclear size in the ventromedial nucleus. After unilateral adrenalectomy, the cell nuclei were enlarged on the contralateral side, and on the ipsilateral side, nuclear shrinkage effect was observed. A unilateral effect in the brain could not be brought about other than by purely neural mechanism. In view of the regulation of DBH activity by both hormonal and neural factors (Chapter I-8), the effect of interrupting the hypothetical neural input into the central nervous system from one adrenal gland on DBH activity in individual sides of the brain stem and the hypothalamus was investigated. No differences could be detected one or two days after unilateral adrenalectomy. Hence the absence of neural input from the adrenal glands could not account for the decreased DBH activity in the brain of adrenalectomized rats.

In conclusion, large dose of corticosterone increased DBH activity, and bilateral adrenalectomy transiently decreased it in the hypothalamus and brain stem of the rat. However, physiological doses of corticosterone did not replete DBH in the adrenalectomized rat. This suggests, although it certainly does not prove, that the feedback effect of glucocorticoids on brain neurons is not at this site.

Ovariectomy in the rat has been shown to change brain norepinephrine content and turnover (Wurtman, 1971, pp. 251-254), and to increase tyrosine hydroxylase activity in the hypothalamus (Beattie et al., 1972; Kizer et al., 1974). No change in DBH activity in the hypothalamus and brain stem of ovariectomized rats with or without estradiol replacement. This observation agrees with the result reported by Kizer et al. (1974).

==== C H A P T E R II - 9 ====

ESTABLISHMENT OF FLUOROMETRIC ASSAY FOR CATECHOLAMINES AND STANDARDIZATION OF THE PROCEDURE

OBJECTIVE (1) To set up fluorometric assay for measuring norepinephrine and dopamine content in rat brain.

(2) To examine the interassay variation of the procedure.

(3) To study the effect of drugs on catecholamine content in the brain.

INTRODUCTION I have demonstrated changes in dopamine-β-hydroxylase activity after manipulation of the adrenocortical system. Since DBH is the enzyme responsible for the conversion of dopamine to norepinephrine in noradrenergic neurons, it is important to assess whether a change in norepinephrine and /or dopamine content in brain tissue is associated with the change in DBH activity.

To determine dopamine and norepinephrine concentrations in brain tissue, I adopted the chemical assay which involves (1) extraction of catecholamines from brain by perchloric acid, (2) purification of catecholamines by adsorption onto aluminum oxide at pH 8.4, (3) elution of catecholamines from aluminum oxide by dilute acid, and (4) determination of norepinephrine and dopamine in acid eluate by differential fluorometry after they are oxidized and subsequently rearranged (trihydroxyindole reaction, or THI) to form strong fluorophores (lutins).

METHODS AND RESULTS

A. Extraction of Catecholamines from Brain Tissue.

A minor modification of the method described by Shellenberger and Gordon (1971) was used.

Reagents. (1) Perchloric acid ($HClO_4$), 70 %, was a product of Mallinckrodt Chemical Works, St. Louis. (2) Sodium metabisulfite ($Na_2S_2O_5$) was purchased from Matheson Coleman & Bell (MCB), Norwood, Ohio.

The perchloric acid solution used for brain homogenization was prepared by adding 1.0 gram sodium metabisulfite and 0.5 gram disodium EDTA to one liter of 0.4 N perchloric acid (86 ml of 70 % perchloric acid to make one liter aqueous solution).

Procedure. The hypothalamus, brain stem and hippocampus were dissected according to the procedure described in Chapter II-4 (pp. 84-85). Those from two rats were pooled correspondingly, weighed and homogenized in 5 ml of the perchloric acid solution. For larger piece of brain tissue, 10 volumes of perchloric acid solution was used. The homogenates were centrifuged at 20,000 x g for 15 minutes. The supernatant (about 5 ml) was decanted into a 15-ml centrifuge tube (conical buttom, screw capped) and kept in freezer before the subsequent step was carried out within 2 days.

B. Isolation of Catecholamines from Brain Homogenate.

Again, the method of Shellenberger and Gordon (1971) was used.

Reagents. (1) Tricine solution was prepared by dissolving 17.9 grams of Tricine (Calbiochem, San Diego, California) and 25 grams of disodium EDTA in one liter of 0.525 N NaOH. (2) The activated aluminum oxide was prepared by treating Aluminum Oxide Woelm (neutral, activity grade I, made by M. Woelm, 3440 Eschwege, West Germany, and distributed by ICN Pharmaceuticals, Inc., Cleveland, Ohio) according to the procedure of Anton and Sayre (1962), and was stored in a desiccator.

Procedure. (1) Tissue extract (about 5 ml) was poured into a 30-ml beaker and into which was added Tricine solution slowly with constant stirring and and monitoring of pH, until the pH of the mixture was brought to 8.4. Approximately 5 ml of Tricine solution was required.

(2) The mixture was transferred back to the 15-ml centrifuge tube into which 300 mg of activated aluminum oxide was added.

(3) The tube was shaken for 10 minutes to facilitate the adsorption of catecholamines onto aluminum oxide. After centrifugation at low speed, the supernatant was discarded and the aluminum oxide was washed with 10 ml of deionized water for two times.

(4) Finally 4 ml of 0.2 N acetic acid solution was added and shaken for 10 minutes to elute catecholamines into the acid. The supernatant was taken in 1-ml aliquots into 5-ml test tube. These samples were capped and kept in refrigerator at 4°C, with the subsequent step carried out within one day.

C. Oxidation of Catecholamines (Trihydroxyindole, THI, reaction).

A minor modification of the procedure described by Chang (1964) was used.

Reagents. (1) 0.1 M EDTA-acetate buffer: Disodium EDTA dihydrate, 37.5 gm, was dissolved in 1 liter of 0.8 M sodium acetate. The pH was adjusted to 7.5 by the addition of NaOH. (2) 1.45 N NaOH solution. (3) 0.1 N Iodine: 0.64 gm iodine was dissolved in 50 ml of absolute ethanol. (4) Alkaline sulfite: 1 ml of fresh sodium sulfite solution (250 mg of anhydrous salt in 1 ml of water) was diluted with 9 ml of 5 N NaOH just prior to use. (5) 5 N Acetic acid solution. (6) Authentic norepinephrine and dopamine were dissolved in 0.02 N HCl and kept in refrigerator. Standards consisted of 0.1 ml solution containing 25, 50, 100 or 200 ng of catechol-

amines as the free bases.

Procedure. (1) To 1 ml of the eluate or standard catecholamine solution, 0.2 ml of 0.1 M EDTA-acetate solution and 0.1 ml of 1.45 N NaOH were added to bring the pH of the mixture to about 6.0.
(2) 0.1 ml of 0.1 N iodine solution was added to oxidize the catecholamines.
(3) After exactly 2 minutes, the oxidation was stopped by adding 0.2 ml of alkaline sulfite; after another 2 minutes 0.2 ml of 5 N acetic acid solution was added.
(4) To determine norepinephrine, the mixture was heated in boiling water bath for exactly 2 minutes and the fluorescence read while the mixture was kept cold, with the activation at 370 mμ and the emission at 480 mμ. To determine dopamine, the mixture was heated in a boiling water bath for three more minutes (total length of heating was 5 minutes) and the fluorescence read while the mixture was kept cold, with the activation at 315 mμ and the emission at 370 mμ. An Aminco spectrophotofluorometer (SPF 125, American Instrument Co., Inc., Silver Spring, Maryland) was used for this purpose.

With each batch of fluorometric assay, reagent blank and standard amounts of catecholamines were run to obtain a standard curve from which absolute amounts of catecholamines in each sample could be calculated. To 1 ml of the eluate from the aluminum oxide, a premixed solution of all the reagents used in the oxidation was added and the fluorescence was read as the tissue blank (Chang, 1964). It was found that the tissue blanks were very close to the reagent blanks.

D. Linearity and Specificity of Trihydroxy Indole (THI) Reaction: Differential Spectrofluorometry for Norepinephrine and Dopamine.

When known amounts of norepinephrine and/or dopamine were run through

THI reaction and fluorescence read at different meter settings (370/480 mμ for norepinephrine and 315/370 mμ for dopamine), differential amounts of fluorescence units could be recorded. A linear relationship, illustrated in Table 9-1 and Fig. 9-1, was always obtained for the standards assayed simultaneously with unknown samples. That interference between norepinephrine and dopamine did not occur was also demonstrated here, indicating that the assay is specific.

TABLE 9-1 Linearity and Specificity of THI Reaction.

Amount of Catecholamines in each tube (ng base)		Fluorescence Readings (Units)	
		N E	D A
N E	D A	(370/480 mμ)	(315/370 mμ)
0	0	2.0	3.0
25	0	23.7	3.6
50	0	46.0	4.6
100	0	90.7	5.8
200	0	174.5	6.7
0	25	2.6	19.4
0	50	3.9	35.8
0	100	4.7	67.8
0	200	5.5	130.0
100	100	94.0	72.2
200	200	185.2	138.5

Calculated from the table :

For NE: 1 fluorescence unit (at 370/480) = 1.12 ng of free NE.

For DA: 1 fluorescence unit (at 315/370) = 1.54 ng of free DA.

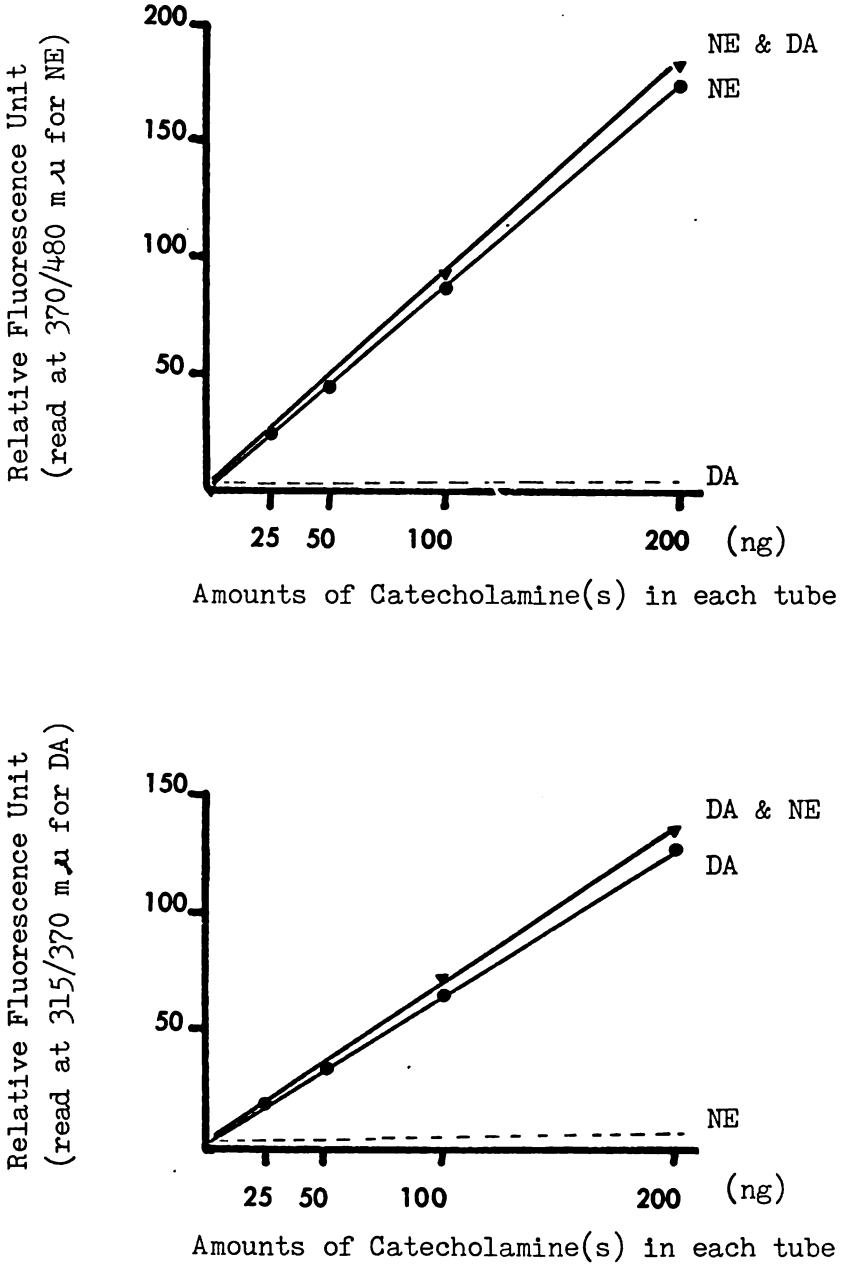


Fig. 9-1 Standard Curves of Norepinephrine and Dopamine (Plotted from the data in Table 9-1).

E. Catecholamine Assay for Brain Extract.

The recovery of norepinephrine and dopamine was estimated after adding known amounts of authentic catecholamines (200 ng each) to 5 ml of brain extract, and running the samples through aluminum oxide step and THI reaction. Table 9-2 illustrates fluorescence readings and the procedure of calculation. Data accumulated indicated that the recovery for norepinephrine ranged between 75-85 %, and that for dopamine ranged between 70-80 %. Results in Table 9-2 again demonstrated that there was no interference between norepinephrine and dopamine after they were run through the extraction and oxidation steps.

TABLE 9-2 Results of Catecholamine Assay for Brain Homogenate: Calculation of the Recovery.

Brain Homogenate	Addition to each Tube		Fluorescence Readings (Units)	
	N E	D A	N E (370/480 mμ)	D A (315/370 mμ)
5 ml	-	-	20	51
5 ml	200 ng	-	65	53
5 ml	-	200 ng	21	81
5 ml	200 ng	200 ng	68	85

1) In 3 ml of acetic acid eluate from aluminum oxide, only 1 ml was run for THI reaction and read for fluorescence, hence the fluorescence readings must be multipled by 3.

2) 1 unit of NE reading (at 370/480 mμ) = 1.12 ng of NE
1 unit of DA reading (at 315/370 mμ) = 1.54 ng of DA (from Table 9-1)

3) Calculation of the recovery:

For NE: 1.12 ng x (65-20) x 3 = 150 ng,
Recovery = 150 ng / 200 ng = 75 %.

For DA: 1.54 ng x (81-51) x 3 = 140 ng,
Recovery = 140 ng / 200 ng = 70 %.

F. Interassay Variation of Catecholamine Determination by Fluorometric Method.

Six samples from a single pool of acid extract of rat brain were run in six different batches of catecholamine assay during a period of 30 days. Norepinephrine and dopamine content, expressed as ug of catecholamine per gram of brain tissue, was shown in Table 9-3. The average concentrations of norepinephrine and dopamine were 0.331 and 0.868 μg/gm, respectively. The coefficient of variation for NE was 3 % and that for DA was 15 %, indicating that the procedure for measuring NE and DA was rather reliable. It was obvious that the method for measuring NE was more accurate than that for measuring DA.

TABLE 9-3 Interassay Variation for Catecholamine Assay.

Six samples from pool of acid extract of rat brain were run in six separate assays during a period of 30 days.

NOREPINEPHRINE CONTENT (μg/gm)	DOPAMINE CONTENT (μg/gm)
0.330	0.800
0.313	1.119
0.330	0.790
0.344	0.912
0.333	0.800
0.335	0.786
MEAN = 0.331	MEAN = 0.868
S.D. = 0.010	S.D. = 0.132
S.E.M. = 0.004	S.E.M. = 0.054

Coefficient of Variation (C.V.) = (S.D. / MEAN) x 100 %

For NE : C.V. = 0.010 / 0.331 = 3 %.

For DA : C.V. = 0.132 / 0.868 = 15 %.

G. Effect of Drugs on Brain Catecholamine Content.

Another way to test the accuracy or the reliability of the assay is to determine brain catecholamine content after the animals are treated with drugs known to alter the metabolism of brain catecholamines. Table 9-4 summarizes the results of three experiments carried out in three species of animals.

Reserpine depletes catecholamine stores by interfering with the uptake and retention of norepinephrine and dopamine in the granular vesicles (Weiner, 1974b). I found that in mice reserpine decreased norepinephrine and dopamine concentrations. U-14624 and FLA 63 are dopamine-β-hydroxylase inhibitors which cause a selective depletion of norepinephrine in the brain with or without a small rise in dopamine content (Johnson et al., 1970; Corrodi et al., 1970). My results in mice, rat and dog confirmed this observation. Alpha-methyl-para-tyrosine is the best inhibitor of tyrosine hydroxylase, and causes a gradual decline of brain dopamine and norepinephrine levels (Spector et al., 1965). My result for rat brain was also consistent with such observation. Pargyline, on the other hand, is a monoamine oxidase inhibitor which prevents the intraneuronal breakdown of dopamine and norepinephrine. The rats given this drug showed an increase in both dopamine and norepinephrine concentrations in the brain. These results which confirmed the previously reported observations clearly indicated that the fluorometric assay I have set up to measure the concentrations of norepinephrine and dopamine is indeed specific and reliable.

DISCUSSION I have set up a method for the assay of norepinephrine and dopamine in the brain. This assay is sensitive to detect the amounts of catecholamines in acid extract of brain sample pooled from discrete regions

of two rats. I have also carried out experiments to test the specificity, accuracy and reliability of the assay.

TABLE 9-4 Effect of Drug Treatment on Brain Catecholamine Content.

Values are means ± S.E.M., expressed in μg of NE or DA per gram of brain tissue. Numbers in parentheses are numbers of animals.

Exp. 1 Male ICR mice, weighing 25 gm, were given i.p. either reserpine, 2 mg/kg, 3 hours before sacrifice or U-14624 (DBH inhibitor), 200 mg/kg, 20 hours before sacrifice. Control mice received no injection. Whole brain catecholamine content was measured.

TREATMENT	NOREPINEPHRINE	DOPAMINE
Control (5)	0.37 ± 0.02	0.80 ± 0.02
Reserpine (5)	0.04 ± 0.03	0.24 ± 0.07
U-14,624 (5)	0.10 ± 0.02	0.90 ± 0.07

Exp. 2 Male Sprague-Dawley rats, weighing 180-200 gm, were given i.p. pargyline, 50 mg/kg, 12 hours before, or α-methyl-p-tyrosine, 250 mg/kg, 3 hours before, or FLA 63 (DBH inhibitor), 10 mg/kg, 4 hours before sacrifice. Whole brain without median eminence was homogenized for catecholamine measurement.

TREATMENT	NOREPINEPHRINE	DOPAMINE
Saline (10)	0.396 ± 0.007	0.582 ± 0.008
Pargyline (8)	0.596 ± 0.009	0.776 ± 0.011
α-MT (10)	0.256 ± 0.012	0.355 ± 0.021
FLA 63 (9)	0.240 ± 0.008	0.760 ± 0.017

TABLE 9-4 (continued)

Exp. 3 Male mongrel dogs, weighing 12-18 kgs, were given i.p. different doses of U-14,624 (DBH inhibitor) suspended in methylcellulose, 18 hours before sacrifice. Dogs were killed by overdosage of pentobarbital and brains were removed immediately. The hypothalamus was dissected and homogenized for catecholamine measurement. Control dogs received only methylcellulose.

INJECTION		NOREPINEPHRINE	DOPAMINE
Methylcellulose	(4)	0.933 ± 0.085	0.313 ± 0.019
U-14624, 200 mg/kg	(3)	0.547 ± 0.024	0.383 ± 0.012
U-14624, 600 mg/kg	(2)	0.315 ± 0.025	0.480 ± 0.020

==== C H A P T E R II - 10 ====

EFFECT OF CORTICOSTERONE TREATMENT AND ADRENALECTOMY ON CATECHOLAMINE CONTENT IN RAT BRAIN

OBJECTIVE (1) To determine the concentrations of norepinephrine and dopamine in the hypothalamus, brain stem and hippocampus of normal rats. (2) To study the change in brain catecholamine content after single or repeated daily injection of large dose of corticosterone. (3) To study the effect of adrenalectomy with or without corticosterone replacement on brain catecholamine content.

INTRODUCTION Although I have demonstrated changes in brain dopamine-β-hydroxylase activity after corticosterone treatment and bilateral adrenalectomy, it was not known whether these would lead to corresponding changes in norepinephrine and/or dopamine content in the brain regions. Using the chemical assay I have adopted and standardized, I measured concentrations of norepinephrine and dopamine in the hypothalamus, brain stem and hippocampus of the rats after adrenalectomy and corticosterone treatment.

METHODS AND RESULTS

A. Catecholamine Content in Brain Regions of Normal Rats.

For the purpose of comparison, mean concentrations of norepinephrine and dopamine in the hypothalamus, brain stem and hippocampus of normal, resting rats, pooled from 5 experiments, are presented in Table 10-1. Brain tissues from the same region of two to three rats were combined for

for one determination. The hypothalamus has the highest norepinephrine content, the brain stem has a moderate content, and the hippocampus has a relatively low concentration of norepinephrine. This correlates very well with the relative amounts of dopamine-β-hydroxylase present in these three brain regions (see Table 5-1, p. 99). Dopamine concentrations in three brain regions also show a similarity in relative magnitude.

TABLE 10-1 Catecholamine Content in Brain Regions of Normal Rats.

Male Sprague-Dawley rats, weighing between 150 and 200 gm, were decapitated immediately after being removed from cages. Brain regions were dissected out and those from 2-3 rats were combined for each determination.
Catecholamine content is expressed as μg per gm of brain tissue. Values are means ± S.E.M., pooled from 5 assays. Numbers in parentheses are numbers of determinations.

BRAIN REGIONS	Number of Animals	NOREPINEPHRINE	DOPAMINE
Hypothalamus	64 (28)	1.818 ± 0.051	0.735 ± 0.018
Brain Stem	62 (27)	0.629 ± 0.006	0.102 ± 0.003
Hippocampus	36 (14)	0.372 ± 0.013	0.045 ± 0.002

B. Effect of Single Large Dose of Corticosterone on Catecholamine Content in Regions of Rat Brain.

Male Sprague-Dawley rats, weighing 150-170 gm, were given subcutaneously corticosterone (10 mg/100 gm) or peanut oil, and were sacrificed 4 hours after injection. Brain regions were dissected out as described. Single hypothalamus, brain stem and hippocampus from one rat were assayed for DBH activity; the hypothalami, brain stems and hippocampi from two rats were combined respectively to be determined for catecholamine content.

TABLE 10-2 Effect of a Single Large Dose of Corticosterone on DBH Activity and Catecholamine Content in Rat Brain.

Male Sprague-Dawley rats, weighing 150-170 gm, were given corticosterone (B), 10 mg/100 gm B.W., or given peanut oil subcutaneously and were sacrificed 4 hours after injection. Control rats received no injection. All values are means ± S.E.M., expressed in nmoles oct./gm/hr for DBH, and in μg/gm for NE and DA. The numbers in parentheses are numbers of animals.

	HYPOTHALAMUS			BRAIN STEM			HIPPOCAMPUS		
INJECT.	DBH	N E	D A	DBH	N E	D A	DBH	N E	D A
None	318.1 ± 7.0 (6)	1.913 ±0.131 (12)	0.818 ±0.032 (12)	221.0 ± 8.3 (6)	0.601 ±0.014 (12)	0.099 ±0.007 (12)	68.6 ±4.0 (6)	0.318 ±0.015 (12)	0.040 ±0.003 (12)
Oil	318.3 ± 6.0 (6)	1.936 ±0.043 (12)	0.843 ±0.039 (12)	222.4 ±12.2 (6)	0.615 ±0.023 (12)	0.096 ±0.003 (12)	65.6 ±4.9 (6)	0.331 ±0.014 (12)	0.038 ±0.003 (12)
B	340.9* ± 3.4 (6)	1.802@ ±0.024 (12)	0.751 ±0.026 (12)	221.4 ± 7.8 (6)	0.580 ±0.016 (12)	0.083@ ±0.004 (12)	64.8 ±4.7 (6)	0.345 ±0.013 (12)	0.043 ±0.002 (12)

* $P < 0.02$ vs oil injected or normal control.

@ $P < 0.03$ vs oil injected.

DBH values in three brain regions after corticosterone treatment have been presented in Table 7-1 (p. 115); in Table 10-2 they are presented together with the values for norepinephrine and dopamine content for a better comparison. As shown in Tables 7-1 and 10-2, DBH activity increased slightly after acute corticosterone treatment. When norepinephrine and dopamine concentrations in the corresponding brain regions were measured for other rats treated in the same way, no parallel changes could be observed. Instead, there was a minor decrease in norepinephrine content in

the hypothalamus and in dopamine content in the brain stem after corticosterone treatment.

C. Effect of Repeated Daily Large Dose of Corticosterone on Catecholamine Content in Regions of Rat Brain.

Male Sprague-Dawley rats, weighing 150-170 gm, were given subcutaneously corticosterone, 10 mg/100 gm/day, or peanut oil, daily for 5 days and were sacrificed 24 hours after the last injection. The hypothalamus, brain stem and hippocampus were dissected out. Single discrete regions from one rat were assayed for DBH activity; those from two rats were combined for the determination of norepinephrine and dopamine content. DBH values in three brain regions after repeated daily corticosterone treatment have already been presented in Table 7-2 (p. 115); in Table 10-3 they are presented together with those of catecholamine content. As shown in Tables 7-2 and 10-3, DBH activity in these regions increased significantly after repeated corticosterone treatment. Norepinephrine and dopamine content in the hypothalamus, on the contrary, decreased markedly whereas the concentrations of the amines in the brain stem did not show any change.

D. Effect of Bilateral Adrenalectomy on Catecholamine Content in Regions of Rat Brain.

The detail of the experimental procedure has been described in Chapter II-8 (p. 119). Rats were bilaterally adrenalectomized or sham-operated under pentobarbital anesthesia and were sacrificed 24 hours or 5 days after operation. Adrenalectomized rats were maintained on normal saline. The hypothalamus, brain stem and hippocampus were dissected out. Single hypothalami, brain stems and hippocampi were assayed for DBH activity; the hypothalami, brain stems and hippocampi from two rats were combined for

TABLE 10-3 Effect of Repeated Large Dose of Corticosterone for 5 Days on DBH Activity and Catecholamine Content in Regions of Rat Brain.

Male Sprague-Dawley rats, weighing 150-170 gm, were given corticosterone (B), 10 mg/100 gm/day, or given peanut oil subcutaneously daily for 5 days and were sacrificed on sixth day. Control rats received no injection. All values are means ± S.E.M., expressed in nmoles oct./gm/hr for DBH, and in μg/gm for NE and DA. The numbers in parentheses are numbers of animals.

	HYPOTHALAMUS			BRAIN STEM			HIPPOCAMPUS
INJECTION	DBH	N E	D A	DBH	N E	D A	DBH
None	301.6 ±11.9 (6)	1.745 ±0.076 (12)	0.075 ±0.027 (12)	203.9 ± 5.8 (6)	0.626 ±0.006 (12)	0.122 ±0.005 (12)	61.3 ±3.5 (6)
Oil, 5 d.	314.0 ± 8.2 (7)	1.344 ±0.171 (12)	0.603 ±0.050 (12)	201.1 ± 6.5 (7)	0.629 ±0.010 (12)	0.121 ±0.003 (12)	64.7 ±2.5 (7)
B, 5 days	346.0* ±10.4 (7)	1.051@ ±0.091 (12)	0.397* ±0.019 (12)	248.5* ± 7.6 (7)	0.608 ±0.011 (12)	0.120 ±0.003 (12)	75.3 ±2.5 (7)

* $P < 0.03$ vs oil injected or normal control.

@ $P < 0.0002$ vs normal control.

the determination of catecholamine concentrations. DBH values in these regions at 24 hours or 5 days after adrenalectomy have already been shown in Table 8-1 (p. 120); in Table 10-4 they are presented with the values for catecholamine content. As shown in Tables 8-1 and 10-4, DBH activity in brain stem decreased slightly 1 day and 5 days after adrenalectomy; the contentin the hypothalamus decreased one day after adrenalectomy, but returned to the normal level after 5 days. In contrast, the concentrations of norepinephrine and dopamine in those regions did not show any

change after bilateral adrenalectomy.

TABLE 10-4 Effect of Adrenalectomy on DBH Activity and Catecholamine Content in Regions of Rat Brain.

Male Sprague-Dawley rats, weighing 150-170 gm, were adrenalectomized (AdX) or sham-operated under pentobarbital anesthesia and were killed 24 hours or 5 days after the operation. Adrenalectomized rats were maintained on normal saline. Control rats did not receive any manipulation. All values are means ± S.E.M., expressed in nmoles oct./gm/hr for DBH, and in μg/gm for NE and DA. The numbers in parentheses are numbers of animals.

	HYPOTHALAMUS			BRAIN STEM			HIPPOCAMPUS		
OPERAT.	DBH	NE	DA	DBH	NE	DA	DBH	NE	DA
A. 24 hours after the operation.									
None	312.4 ± 5.7 (6)	1.932 ±0.060 (12)	0.715 ±0.028 (12)	211.4 ± 4.2 (6)	0.622 ±0.019 (12)	0.092 ±0.002 (12)	66.1 ±0.6 (6)	0.371 ±0.018 (12)	0.041 ±0.002 (12)
Sham	309.7 ± 4.0 (6)	1.936 ±0.068 (12)	0.702 ±0.037 (12)	224.3 ± 4.7 (6)	0.605 ±0.009 (12)	0.081 ±0.002 (12)	63.7 ±1.2 (6)	0.335 ±0.011 (12)	0.039 ±0.001 (12)
AdX	281.8* ± 6.7 (6)	1.893 ±0.095 (12)	0.710 ±0.020 (12)	184.9* ± 9.2 (6)	0.615 ±0.015 (12)	0.087 ±0.004 (12)	63.1 ±3.4 (6)	0.341 ±0.012 (12)	0.039 ±0.004 (12)
B. 5 days after the operation.									
None	312.4 ±13.3 (6)	1.878 ±0.062 (12)	0.682 ±0.015 (12)	221.7 ±14.8 (6)	0.626 ±0.011 (10)	0.090 ±0.040 (10)			
Sham	320.7 ± 7.9 (7)	1.949 ±0.027 (12)	0.692 ±0.014 (12)	248.2 ±11.5 (7)	0.648 ±0.013 (10)	0.094 ±0.020 (10)			
AdX	310.3 ± 8.7 (7)	1.969 ±0.062 (14)	0.692 ±0.016 (14)	188.2@ ± 8.7 (7)	0.645 ±0.012 (8)	0.095 ±0.030 (8)			

* $P < 0.01$ vs sham-operated or unoperated control.
@ $P < 0.001$ vs sham-operated.

E. Effect of Corticosterone Replacement in Adrenalectomized Rats on Catecholamine Content in Regions of Rat Brain.

Rats were bilaterally adrenalectomized or sham-operated under pentobarbital anesthesia two days before sacrifice. Adrenalectomized rats were maintained on normal saline and were given either various dose of corticosterone or peanut oil subcutaneously at 4 hours or 24 hours before sacrifice. The hypothalamus and brain stem were dissected out. Single hypothalami and brain stems were assayed for DBH activity; the hypothalami and brain stems from two rats were combined respectively for the determination of catecholamine content. DBH values in adrenalectomized rats with or without corticosterone replacement have already been summarized in Table 8-2; in Table 10-5 they are presented again with those of catecholamine content. As shown in Table 8-2, DBH activity DBH activity in the two brain regions decreased by 15-25 % two days after adrenalectomy. It is obvious in Tables 8-2 and 10-5 that decreased DBH activity in adrenalectomized rats was not restored after corticosterone replacement. Similarly, no change in catecholamine concentrations could be observed in those rats.

DISCUSSION Although large dose of corticosterone in rats with intact adrenals led to increased DBH activity, catecholamine concentrations in the hypothalamus decreased. These two findings seem conflicting. In view of lack of study on this aspect, no explanation for the decrease in norepinephrine content can be obtained. Glucocorticoids have been shown to decrease the uptake of norepinephrine into the heart (Iversen and Salt, 1970) and increase the uptake into brain slices (Maas and Mednieks, 1971). However, it is impossible to relate these findings to the observation of decreased norepinephrine content in the hypothalamus after corticosterone treatment.

TABLE 10-5 Effect of Corticosterone Replacement in Adrenalectomized Rats on DBH Activity and Catecholamine Content in Regions of Rat Brain.

Male Sprague-Dawley rats, weighing 150-170 gm, were adrenalectomized under pentobarbital anesthesia 2 days before sacrifice. They were all maintained on normal saline. Corticosterone (B) in various dosages or peanut oil was given subcutaneously 4 hours or 24 hours prior to sacrifice. All values are means ± S.E.M., expressed in nmoles oct./gm/hr for DBH, and μg/gm for NE and DA. The numbers in parentheses are numbers of animals.

	HYPOTHALAMUS			BRAIN STEM		
INJECTION	DBH	N E	D A	DBH	N E	D A
1. Injection at 4 hours before sacrifice.						
Oil	272.8 ± 6.4 (5)	1.630 ±0.060 (10)	0.658 ±0.030 (10)	179.7 ± 2.9 (5)	0.596 ±0.020 (10)	0.098 ±0.010 (10)
B, 5 mg/kg	274.4 ±10.4 (5)	1.670 ±0.070 (10)	0.632 ±0.010 (10)	179.1 ± 4.4 (5)	0.619 ±0.010 (10)	0.091 ±0.003 (10)
B, 10 mg/kg	267.0 ±10.4 (5)	1.715 ±0.030 (10)	0.666 ±0.020 (10)	182.6 ± 6.2 (5)	0.605 ±0.020 (10)	0.095 ±0.002 (10)
B, 20 mg/kg	283.1 ± 7.1 (5)	1.658 ±0.030 (10)	0.634 ±0.010 (10)	180.4 ± 7.6 (5)	0.602 ±0.010 (10)	0.098 ±0.003 (10)
2. Injection at 24 hours before sacrifice.						
Oil	277.6 ± 7.6 (5)	1.622 ±0.040 (10)	0.685 ±0.030 (10)	178.2 ± 8.1 (5)	0.596 ±0.010 (10)	0.094 ±0.002 (10)
B, 5 mg/kg	268.0 ± 9.1 (5)	1.625 ±0.090 (10)	0.672 ±0.040 (10)	180.8 ± 7.5 (5)	0.593 ±0.010 (10)	0.093 ±0.006 (10)
B, 10 mg/kg	280.4 ± 9.2 (6)	1.591 ±0.080 (10)	0.697 ±0.030 (10)	177.7 ± 6.3 (6)	0.619 ±0.010 (10)	0.094 ±0.005 (10)
B, 20 mg/kg	273.8 ± 9.0 (6)	1.691 ±0.090 (10)	0.676 ±0.030 (10)	181.7 ± 3.0 (6)	0.614 ±0.010 (10)	0.098 ±0.006 (10)

DISCUSSION (continued)

Adrenalectomy has been shown to alter the turnover of norepinephrine without changing its static concentration in peripheral sympathetic neurons and in the brain (Chapter I-10, pp. 55-58). My study confirmed this observation, showing that no change in catecholamine content could be found in brain regions of adrenalectomized rats with or without corticosterone replacement. It is concluded from these studies that under certain conditions in which minor changes in DBH activity could be observed, it is not necessarily true that we can obtain parallel changes in the concentrations of norepinephrine in same brain regions. Because the steady-state concentration of a given biogenic amine is the net sum of continuous production and breakdown of that amine in the neuronal structures, lack of change in the concentration of the amine does not exclude the possibility of an altered neuronal metabolism.

==== C H A P T E R II - 11 ====

ISOTOPE METHOD TO STUDY THE FORMATION OF NOREPINEPHRINE IN BRAIN REGIONS OF LIVING RATS

OBJECTIVE (1) To implant a chronic cannula for the injection of radioactive catecholamines into the third or lateral cerebral ventricle of rat.
(2) To separate norepinephrine from dopamine by cation-exchange chromatography through a Dowex resin column.
(3) To measure the accumulation of tritiated norepinephrine after the injection of tritiated dopamine into the third ventricle, as an index of *in vivo* DBH activity.
(4) To examine the effect of drugs on the formation of radioactive norepinephrine in the brain.

INTRODUCTION *In vitro* techniques for measuring the activity of dopamine-β-hydroxylase in various tissues have offered valuable insight into the factors regulating the amount or activity of this enzyme, as well as the processes concerning the biosynthesis and release of catecholamines in the peripheral and central nervous system. Using one of those techniques I was able to obtain changes in DBH activity after endocrinological manipulation. However, procedures for assessing the capacity for synthesizing norepinephrine in the living, behaving animal are more important. With the documented presence of endogenous inhibitors of DBH in the brain and the obligatory requirement for cofactors for optimal enzyme activity, a knowledge of *in vivo* activity of this enzyme seems important. Furthermore, it appears that the assessment of the dynamic turnover of the amine *in vivo*

will provide more information about neuronal activity than the measurement of amine content in the brain tissue (for discussion, see Neff et al., 1971). Among a wide variety of methods to estimate the synthesis and turnover rates of norepinephrine in the brain, I adopted the one reported by Stolk (1973) to determine the relative magnitude of the *in vivo* activity of dopamine-β-hydroxylase in rat brain. The procedure involves the intraventricular injection through an implanted cannula of known amount of radioactive dopamine and measurement of the amount of radioactive norepinephrine accumulated in the brain at timed intervals thereafter.

METHODS AND RESULTS

A. Implantation of Cannula Guide for Intraventricular Injection.

Permanent cannula guide was implanted stereotaxically in the third ventricle according to de Groot's atlas (1959). The tip of the cannula was introduced to a position 1.5 mm above the floor of the third ventricle in the arcuate-median eminence region (Fig. 11-1). The cannula guides were polished pieces of 23-gauge, thin-wall stainless steel tubing, 16 mm long (HTX-23TW, OD .025", ID .017", Small Parts Inc., Miami, Florida). Under pentobarbital anesthesia, rats were put on a stereotaxic apparatus and the cannulas were implanted through a skull window. Fast-curing dental cement anchored on small screws secured in the skull served to hold the cannulas in place. An inner plug of 28-gauge wire (OD .014") was inserted in the cannula. When indian ink was injected into the third ventricle of some rats through the cannula guide 7 days after implantation, the wall of the ventricular system was coated with dark ink as illustrated in Fig. 11-2. It is obvious under the microscope that the tip of the cannula guide is much larger than the width of the third ventricle.

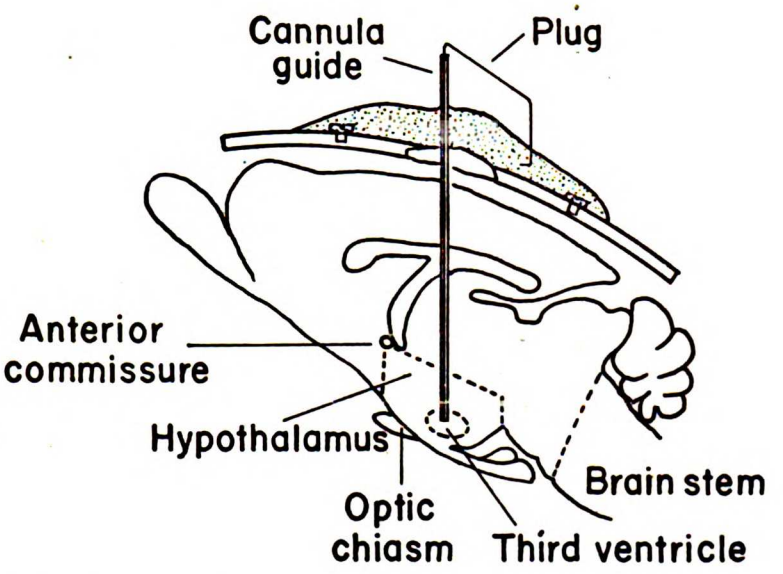


Fig. 11-1 Cannula Implant in the Third Ventricle of the Rat.

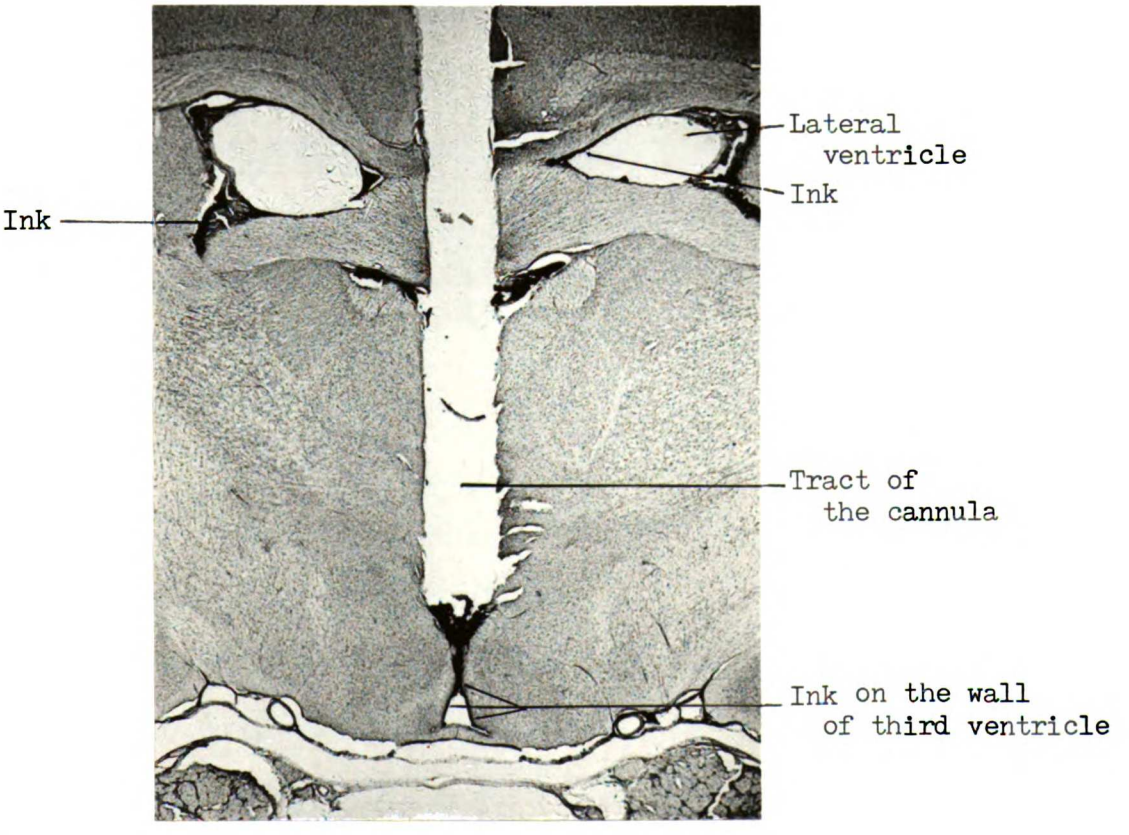


Fig. 11-2 Coronal Section through the Hypothalamus of the Rat Bearing a Ventricular Cannula. Injected indian ink is in ventricles.

When the cannula was properly placed in the midline of the brain it would invariably hit the third ventricle even though there was no spontaneous flow of cerebrospinal fluid out of the cannula immediately after implantation. The rats were used 7 days after the surgery. They all appeared normal at that time.

For the injection into lateral ventricle, the 23-gauge thin-wall cannula was placed 1.5 mm above the roof of right lateral ventricle and was secured in place by dental cement and screws. No inner plug was necessary in such instance. The needle for injecting radioactive catecholamines into the lateral ventricle was introduced 2.5 mm beyond the tip of the cannula. At the time of sacrifice 7 days after implantation, there was no pathologic change of the meninges and brain parenchyme.

B. Cation Exchange Chromatography for the Separation of Norepinephrine and Dopamine.

Tritiated dopamine (3,4-dihydroxyphenylethylamine [ethyl-1-^{3}H (N)], specific activity 10 Ci/mmole) was purchased from New England Nuclear. L-Norepinephrine-7-^{3}H (specific activity 7.3 Ci/mmole) was a product of Amersham/Searle Corporation. Cation exchange resin (Dowex 50 W-X4, 200-400 mesh, hydrogen form) was purchased from Bio-Rad Laboratories, Richmond, California.

A 0.6 x 4.0 cm Dowex 50W-X4 column in sodium form, pH 6.0, was prepared according to the method of Snyder and Taylor (1972). Acid solutions containing known amounts of tritiated norepinephrine and/or dopamine were applied to the columns after the pH of the solutions has been adjusted to 6.0. Amines with catechol nucleus were adsorbed on the resin and were then eluted differentially with increasing concentrations of hydrochloric acid. The radioactivity in acid eluates was determined in a counter after

0.5 ml aliquots of eluates were mixed with 10 ml aqueous scintillation counting fluid (one liter of Triton X-100 to be added to two liters of toluene solution containing 10 gm of PPO). Fig. 11-3 illustrates elution curves for norepinephrine and dopamine. It was shown that norepinephrine could be eluted with 8 ml of 1 N HCl after 4 ml of 1 N HCl had been passed through the column. Dopamine was then eluted with 8 ml of 2 N HCl after 1 ml of 2 N HCl had been passed. Minute amounts of normetanephrine may be present in the same fraction as dopamine. Overlap of amines into adjacent fractions was found to be less than 10 %, and the recovery through the resin column was about 80 %.

C. Time Course of ^{3}H-Norepinephrine Formation in Rat Brain Regions after the Injection of ^{3}H-Dopamine into the Third Ventricle.

Male Sprague-Dawley rats, weighing 180-200 gm, were implanted with stainless steel cannula in the third ventricle under pentobarbital anesthesia 7 days prior to sacrifice. On the day of experiment, each rat received 1.5 μCi/200 gm B.W. of ^{3}H-dopamine (23 ng, or 10^{6} CPM) in a volume of 10 μl into the third ventricle through the cannula guide. Rats were sacrificed at timed intervals after injection. The hypothalamus and brain stem from one rat were dissected out and homogenized in 5 ml of 0.4 N perchloric acid solution, respectively. After the pH of the supernatant was adjusted to 8.4, it was shaken with aluminum oxide to adsorb catecholamines, which were subsequently eluted with 4 ml of 0.2 N acetic acid (for detail, see Chapter II-9, pp. 129-130). A portion (1 ml) of the acid eluate was used to estimate endogenous norepinephrine and dopamine concentrations by trihydroxyindole method as described. Another portion (2 ml) of the acid eluate was adjusted to pH 6.0 and passed through the Dowex resin column to separate norepinephrine and dopamine. Radioactivity for norepinephrine

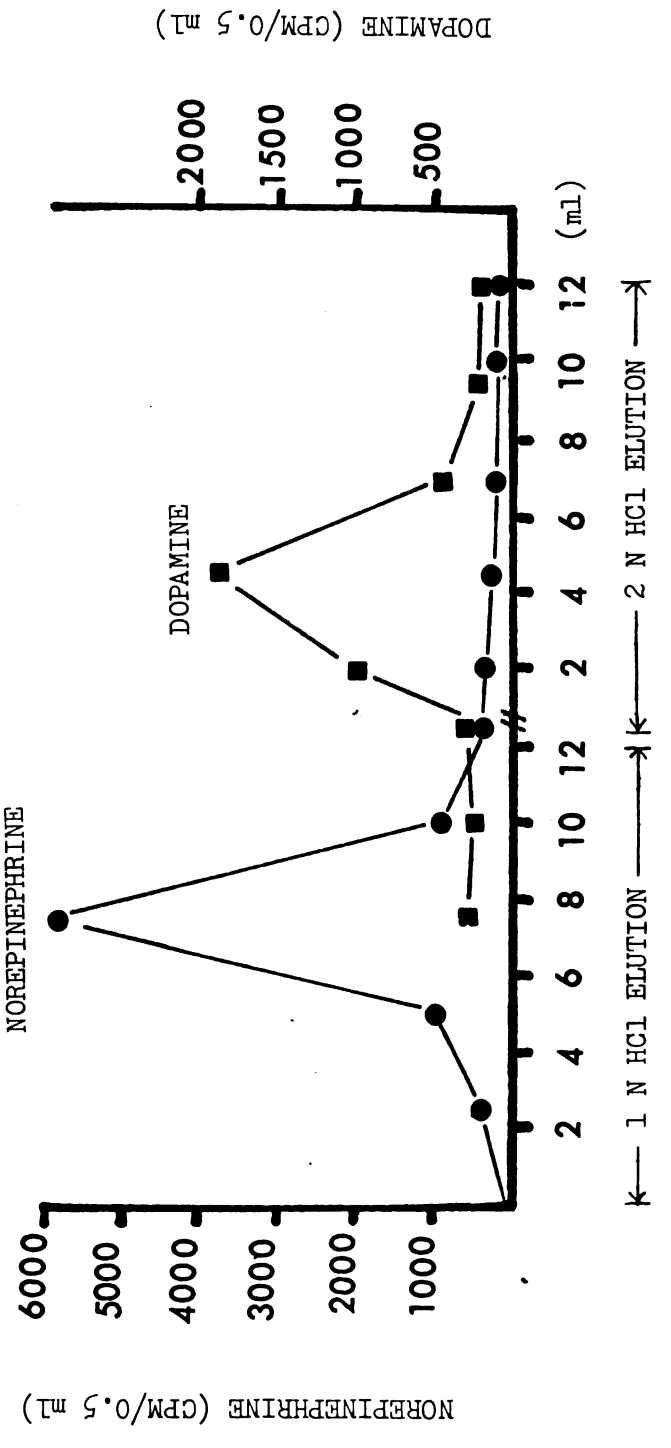


Fig. 11-3 Elution Curves for Norepinephrine and Dopamine from Dowex 50W-X4.

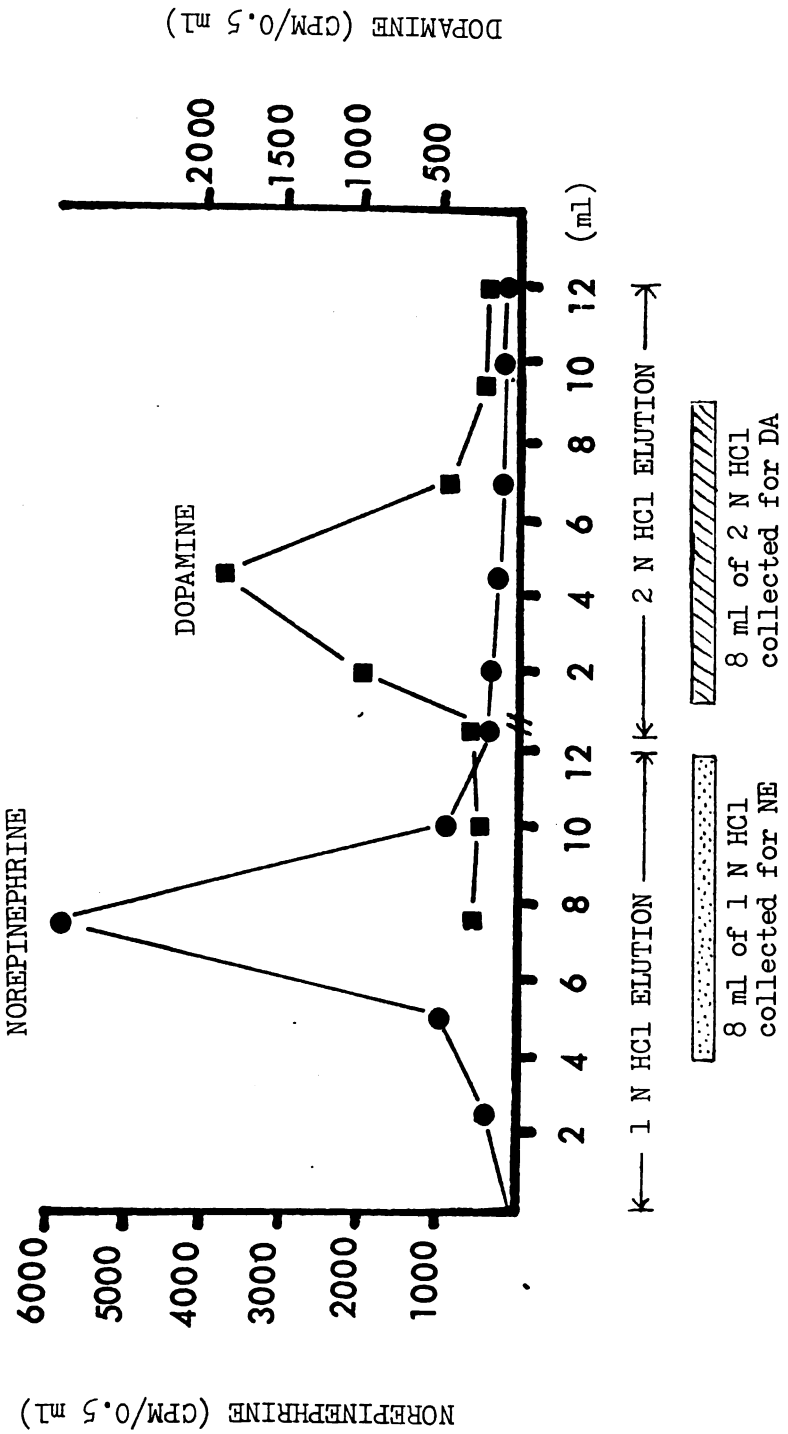


Fig. II-3 Elution Curves for Norepinephrine and Dopamine from Dowex 50W-X4.

or dopamine in the eluate from resin column was determined in a liquid scintillation counter. Taking into account the counting efficiency, recovery of chromatographic procedure and fraction of the aliquot used, and knowing the specific activity of the injected catecholamines, the radioactivity readings were converted into the amount of tritiated catecholamines (ng) present in per unit weight of brain tissue. It should be noted that the amount of radioactive norepinephrine present in the brain at a particular time is the net result of synthesis and metabolism; hence the term "accumulation" is more appropriate than "synthesis" or "formation". Moreover, dopamine injected into the brain can be either taken up by noradrenergic neurons to be converted to norepinephrine, or taken up by dopaminergic neurons to be metabolized via its own catabolic pathways. Therefore, the labeled dopamine left in the brain tissue may be in both noradrenergic and dopaminergic neurons.

Table 11-1 summarizes the results of the time course study. Norepinephrine accumulated in the hypothalamus from the labeled precursor reached maximum levels by 40 minutes after the injection. The amount of labeled norepinephrine present or that of labeled dopamine left in the hypothalamus only constituted a small fraction (about 3 %) of the injected labeled dopamine. The amount of labeled dopamine left in the hypothalamus declined steadily throughout the period studied. When the ratios of ^{3}H-NE to ^{3}H-DA were calculated for each time point, they appeared to be increasing with time for up to two hours. However, this probably does not represent a continuous formation of norepinephrine from its radioactive precursor; rather, it is a result of the steady decrease in the amount of labeled dopamine primarily due to its breakdown via catabolic pathways.

In view of the leveling off after 40 minutes in the amount of hypothalamic norepinephrine, it is conceivable that the rate of biosynthesis of

norepinephrine from the radioactive precursor exceeded that of its breakdown. In looking for an index for the in vivo activity of dopamine-β-hydroxylase, it seemed appropriate to study the accumulation of newly synthesized norepinephrine in the hypothalamus by 40 minutes after the introduction of labeled dopamine into the cerebral ventricle.

In the brain stem, the amounts of norepinephrine accumulated and dopamine left were lowered than those in the hypothalamus. This was consistent with the observation that there are low concentrations of catecholamines in the brain stem as revealed by fluorometric and histochemical studies. There is also higher DBH activity in the hypothalamus than in the brain stem, measured by in vitro assay technique. However, the hypothalamus was exposed to a higher concentration of radioactive dopamine after it was injected into the third ventricle.

D. Effect of 6-Hydroxydopamine and U-14,624 on the Conversion of ^{3}H-Dopamine to ^{3}H-Norepinephrine.

In order to study the validity of the procedure for assessing in vivo DBH activity in the brain, drugs such as 6-hydroxydopamine, which destroys noradrenergic endings, and U-14,624, which is a DBH inhibitor, were administered to the rats and their effect on the conversion of labeled dopamine to norepinephrine was examined. Rats were implanted with a cannula in the third ventricle 7 days prior to sacrifice. 6-Hydroxydopamine, 200 μg/rat per day in a volume of 10 μl, was injected through the cannula guide into the third ventricle at 48 and 24 hours before sacrifice. U-14,624, 200 mg/kg, was suspended in methylcellulose to be injected intraperitoneally into the rats at 20 hours before sacrifice. Each rat received 1.5 μCi/200 gm B.W. of ^{3}H-dopamine (23 ng, or 10^{6} CPM) in a volume of 10 μl into the third ventricle through the cannula guide, and was sacrificed at 40

minutes after injection. Radioactive norepinephrine and dopamine in the hypothalamus and brain stem were determined according to the procedure described above.

TABLE 11-1 Time Course of Tritiated Norepinephrine (^{3}H-NE) Formation in Regions of Rat Brain after Tritiated Dopamine (^{3}H-DA) Injection into the Third Ventricle.

Male Sprague-Dawley rats, weighing 180-200 gm, were implanted with a stainless steel cannula in the third ventricle under pentobarbital anesthesia 7 days prior to sacrifice. Each rat received 1.5 μCi/200 gm B.W. of ^{3}H-DA (23 ng, or 10^6 CPM) in a volume of 10 μl into third ventricle, and was sacrificed at various time after injection. Values are means ± S.E.M. of 4 rats.

	TIME AFTER ^{3}H-DA INJECTION			
HYPOTHALAMUS	20 min	40 min	60 min	120 min
^{3}H-NE (ng/gm brain)	7.66 ±0.98	9.28 ±1.11	9.52 ±0.71	9.03 ±1.20
^{3}H-DA (ng/gm brain)	10.65 ±0.50	7.73 ±1.51	4.19 ±0.82	3.23 ±0.34
Ratio of ^{3}H-NE/^{3}H-DA	0.72 ±0.07	1.34 ±0.27	2.41 ±0.23	2.85 ±0.41
BRAIN STEM				
^{3}H-NE (ng/gm brain)	2.49 ±0.22	2.39 ±0.38	1.83 ±0.32	1.39 ±0.24
^{3}H-DA (ng/gm brain)	2.30 ±0.29	1.66 ±0.32	1.20 ±0.15	0.81 ±0.21
Ratio of ^{3}H-NE/^{3}H-DA	1.14 ±0.16	1.59 ±0.33	1.67 ±0.43	2.09 ±0.55

Table 11-2 summarizes the results of two experiments. In the hypothalamus, prior treatment with 6-hydroxydopamine intraventricularly caused a 90 % reduction in the formation of labeled norepinephrine. The uptake and retention of ^{3}H-dopamine was reduced to half of that of the control due to destruction of noradrenergic endings and possibly dopaminergic endings as well. In the brain stem similar findings were also obtained. These results were consistent with the finding of reduced DBH activity in regions of rat brain after intraventricular 6-hydroxydopamine treatment. Conversion of ^{3}H-dopamine to ^{3}H-norepinephrine in the hypothalamus was inhibited by pretreatment with U-14,624. On a percentage basis, the inhibition was about 50 %, which was comparable to the magnitude of reduction in the endogenous norepinephrine content after U-14,624 treatment (Table 9-4, pp. 137-138). Levels of ^{3}H-dopamine remaining in the hypothalamus of treated animals were two times that of the controls, indicating that the effect of U-14,624 was specific in the blockade of DBH step without the damage to neuronal endings.

DISCUSSION The technique described in the present study was adapted from the technique of Stolk (1973) and was designed to assess the activity of brain DBH *in vivo*. Despite several shortcomings inherent to this technique in particular (Stolk, 1973) and to the turnover studies in general (Weiner, 1974a), the primary objective of this study appeared to be validated. The time course data in Table 11-1 revealed that the rate of norepinephrine accumulation from injected dopamine in the hypothalamus reached its peak by 40 minutes; thereafter the catabolism of the newly synthesized norepinephrine became apparent. The effect of 6-hydroxydopamine pretreatment on *in vivo* DBH activity shown in Table 11-2 was in good agreement with results obtained from the *in vitro* assay technique.

TABLE 11-2 Effect of Pretreatment with 6-Hydroxydopamine or U-14,624 on the Formation of ^{3}H-NE from ^{3}H-DA.

Male Sprague-Dawley rats, weighing 180-200 gm, were implanted with a stainless steel cannula in the third ventricle 7 days prior to sacrifice. 6-Hydroxydopamine, 200 µg/rat in a volume of 10 µl, was injected into the third ventricle through the cannula guide at 48 and 24 hours before sacrifice. U-14,624 (DBH inhibitor), 200 mg/kg, was suspended in methylcellulose and injected i.p. into rats at 20 hours before sacrifice. Each rat received 1.5 µCi/200 gm B.W. of ^{3}H-DA (23 ng, or 10^{6} CPM) in a volume of 10 µl into the third ventricle, and was sacrificed at 40 minutes after injection. The values are means ± S.E.M. The numbers in parentheses are numbers of animals.

	HYPOTHALAMUS			BRAIN STEM		
INJECTION	^{3}H-NE (ng/gm)	^{3}H-DA (ng/gm)	^{3}H-NE/^{3}H-DA	^{3}H-NE (ng/gm)	^{3}H-DA (ng/gm)	^{3}H-NE/^{3}H-DA
None	9.28 ±1.11 (4)	7.73 ±1.51 (4)	1.34 ±0.27 (4)	2.39 ±0.38 (4)	1.66 ±0.32 (4)	1.59 ±0.33 (4)
6-OHDA	0.92 ±0.11 (5)	4.18 ±0.37 (5)	0.24 ±0.05 (5)	0.34 ±0.03 (5)	0.78 ±0.11 (5)	0.45 ±0.04 (5)
None	8.69 ±0.80 (7)	6.52 ±0.62 (7)	1.36 ±0.13 (7)			
U-14,624	4.05 ±0.66 (5)	14.41 ±1.40 (5)	0.30 ±0.07 (5)			

It should also be noted that the degree of reduction in in vivo DBH activity paralleled the extent of endogenous norepinephrine depletion after 6-hydroxydopamine. U-14,624 inhibits the activity of DBH and leads to a depletion of endogenous norepinephrine content in the brain. The decreased formation of labeled norepinephrine from dopamine coincides well with those observations.

In conclusion, all of the findings suggested that the conversion of injected dopamine to norepinephrine occurred primarily within functional noradrenergic neurons catalyzed by DBH. Thus the procedure could be applied to assess the change in the relative magnitude of in vivo DBH activity after manipulation of the adrenocortical system.

==== C H A P T E R II - 12 ====

EFFECT OF CORTICOSTERONE TREATMENT AND ADRENALECTOMY ON THE FORMATION AND DISAPPEARANCE OF ^{3}H-NOREPINEPHRINE IN RAT HYPOTHALAMUS

OBJECTIVE (1) To study the effect of corticosterone treatment or adrenalectomy on the formation of ^{3}H-norepinephrine in the hypothalamus from ^{3}H-dopamine injected into the third ventricle or right lateral ventricle.

(2) To study the effect of adrenalectomy on the disappearance of ^{3}H-NE in the hypothalamus after it was injected into right lateral ventricle.

INTRODUCTION In the hypothalamus and brain stem of the rat, I have demonstrated a slight increase in DBH activity after corticosterone treatment and a decrease in its activity after bilateral adrenalectomy. If dopamine-β-hydroxylase has an important regulatory role in the biosynthesis of norepinephrine, one might expect that adrenalectomy and/or corticosterone treatment would change the rate of synthesis of norepinephrine from dopamine which would manifest itself by an alteration in the rate of norepinephrine turnover, and possibly by a change in the static tissue content as well. The second part of this prediction was not supported by the data in Chapter II-10; no parallel change in norepinephrine content in the hypothalamus and brain stem was observed. However, a discrepancy between the turnover rate of norepinephrine and its steady-state concentration has been reported in quite a few instances (see Chapter I-9). Obviously, the examination of the dynamic turnover in the living rat became essential in this respect. Using the method described in the last chapter

to determine the accumulation of hypothalamic ^{3}H-norepinephrine newly synthesized from ^{3}H-dopamine injected cerebral ventricle, the synthetic capacity of noradrenergic neurons was estimated as an index of the relative magnitude of the in vivo DBH activity. Because the amount of radioactive norepinephrine accumulated during the period studied is the net result of formation and destruction, it is possible that the change in the amount of labeled norepinephrine is merely due to a change in its destruction. In order to rule out this possibility, an additional study was undertaken to follow the disappearance of ^{3}H-norepinephrine in the hypothalamus after it was introduced into the lateral ventricle of the adrenalectomized rat.

METHODS AND RESULTS

A. Effect of Adrenalectomy or Corticosterone Treatment on the Formation of ^{3}H-NE from ^{3}H-DA Injected into the Third Ventricle of the Rat.

Two experiments were done for this study. In the first one, a stainless steel cannula was implanted into the third ventricle of male Sprague-Dawley rats, weighing 180-200 gm, under pentobarbital anesthesia 7 days before sacrifice. Adrenalectomy or sham operation was carried out two days before sacrifice under pentobarbital. Adrenalectomized rats were maintained on normal saline, and some were given replacement of corticosterone, 2 mg/100 gm B.W., subcutaneously at 48, 24 and 4 hours before sacrifice. Some rats without adrenalectomy or sham operation were injected with corticosterone (10 mg/100 gm) or peanut oil s.c. at 4 hours before sacrifice. Each rat received 1.5 μCi/200 gm B.W. of ^{3}H-DA (23 ng) into the third ventricle through the cannula guide and was sacrificed 40 minutes after injection. The amount of radioactive norepinephrine present in the hypothalamus of each rat was determined according to the procedure

TABLE 12-1 Effect of Adrenalectomy or Corticosterone Treatment on the Formation of ^{3}H-NE from ^{3}H-DA Injected into the Third Ventricle.

Exp. 1 A stainless steel cannula was implanted into the third ventricle of male rats, weighing 180-200 gm, under pentobarbital anethesia 7 days before sacrifice. Adrenalectomy or sham operation was carried out 2 days before sacrifice under pentobarbital. Adrenalectomized rats were maintained on normal saline, and some were treated with corticosterone, 2 mg/100 gm, s.c. at 48, 24 and 4 hours before sacrifice. Some rats without adrenalectomy or sham operation also received corticosterone, 10 mg/100 gm, or peanut oil s.c. at 4 hours before sacrifice. Each rat received 1.5 μCi/200 gm of ^{3}H-DA (23 ng) into the third ventricle and was sacrificed 40 minutes after injection. The values are means ± S.E.M., and the numbers in parentheses are numbers of animals.

OPERATION	INJECTION		^{3}H-NE formed in 40 min. (ng/gm hypothalamus)
None	None	(7)	8.69 ± 0.80
Sham	None	(5)	8.48 ± 0.79
AdX	None	(9)	7.58 ± 0.46
AdX	B, 2 mg/100 gm	(5)	8.25 ± 1.19
None	Oil, 4 hrs	(4)	8.97 ± 0.55
None	B, 10 mg/100 gm 4 hrs.	(5)	7.87 ± 0.62

Exp. 2 Each rat, weighing 160-180 gm, received 1.0 μCi (15.5 ng) of ^{3}H-DA into the third ventricle and was sacrificed 20 minutes after injection. Adrenalectomy or sham operation was performed 2 days before sacrifice. Adrenalectomized rats were maintained on normal saline and some were given corticosterone, 1 mg/100 gm, s.c. at 48, 24 and 4 hours before sacrifice. Values are means ± S.E.M., and the numbers in parentheses are numbers of animals.

OPERATION	INJECTION OF B		^{3}H-NE (ng/gm)	^{3}H-DA (ng/gm)	^{3}H-NE/^{3}H-DA
Sham	None	(7)	6.25 ± 0.47	6.64 ± 0.82	0.98 ± 0.06
AdX	None	(7)	6.32 ± 1.05	6.06 ± 1.02	1.05 ± 0.03
AdX	Yes	(6)	5.93 ± 1.05	5.39 ± 0.96	1.18 ± 0.08

described in Chapter II-11. As shown in Table 12-1, no significant change in the accumulation of ^{3}H-NE could be observed in the hypothalmus of the rat after either sham operation, adrenalectomy, or large dose of corticosterone treatment.

In the second experiment, each rat, weighing 160-180 gm, received 1.0 μCi/200 gm B.W. of ^{3}H-DA (15.5 ng) into the third ventricle through the cannula guide and was sacrificed 20 minutes after injection. Adrenalectomy or sham operation was performed two days before sacrifice. Adrenalectomized rats were maintained on normal saline, and some were given corticosterone, 1 mg/100 gm, s.c. at 48, 24 and 4 hours before sacrifice. The amounts of radioactive norepinephrine and dopamine present in the hypothalamus was determined according to the procedure described. Examination of the results shown in the lower part of Table 12-1 indicated that there was again no change in the amount of ^{3}H-NE or ^{3}H-DA, nor the change in the ratio of ^{3}H-NE to ^{3}H-DA, in the hypothalamus of adrenalectomized rats with or without corticosterone replacement.

B. Effect of Adrenalectomy on the Formation of ^{3}H-NE in Rat Hypothalamus from ^{3}H-DA Injected into Right Lateral Ventricle.

The introduction of radioactive doapmine into the third ventricle might have caused changes in the structure and function of neural elements in the hypothalamus which might be responsible for the lack of change in the formation of radioactive norepinephrine in that region of the brain after adrenalectomy. To overcome this problem, ^{3}H-DA was introduced into the lateral ventricle which is reasonably far from the hypothalamus. In such case, any artifact due to the injection procedure could be eliminated.

For the injection into lateral ventricle, a 23-gauge thin-wall tubing was placed 1.5 mm above the roof of the anterior horn of right lateral

ventricle of male Sprague-Dawley rats, weighing 160-180 gm, under pentobarbital anesthesia 7 days prior to sacrifice. Adrenalectomy or sham operation was carried out 2 days before sacrifice. Adrenalectomized rats were maintained on normal saline. The needle for injecting tritiated dopamine into the lateral ventricle was introduced 2.5 mm beyond the tip of the cannula guide. On the day of experiment, each rat received 3.7 μCi/200 gm B.W. of ^{3}H-DA (57 ng) in a volume of 10 μl into the right lateral ventricle and was sacrificed at timed intervals after injection. The hypothalamus was dissected out from each rat brain, its norepinephrine and dopamine content measured by fluorometric method, and amounts of radioactive norepinephrine and dopamine determined (Chapter II-11). The specific activity of tritiated norepinephrine was calculated by dividing the amount of ^{3}H-NE (ng of ^{3}H-NE per gm of hypothalamus) by its endogenous content (μg of NE per gm of hypothalamus). The specific activity of dopamine was calculated in the same way.

Table 12-2 summarizes all the data obtained in this study. Again it demonstrates no significant change in the amount of ^{3}H-NE or ^{3}H-DA in the hypothalamus after adrenalectomy, nor is there any change in endogenous content or specific activity. However, if the ^{3}H-NE which accumulated in the hypothalamus during any interval was corrected for the specific activity of ^{3}H-DA during that period, in analogy to the treatment of the data by Sedvall et al. (1968), Zigmond and Wurtman (1970) and Azmitia et al. (1970), a change after adrenalectomy became apparent. A tendency of increased conversion of per unit of dopamine to norepinephrine existed in the hypothalamus of adrenalectomized rats, with the difference of the ratio between two groups of rats reaching statistical significance ($P < 0.05$) 20 minutes after ^{3}H-DA was introduced into the lateral ventricle.

TABLE 12-2 Effect of Adrenalectomy on the Formation of ^{3}H-NE in Rat Hypothalamus from ^{3}H-DA Injected into the Lateral Ventricle.

A stainless steel cannula guide was implanted above the roof of right lateral ventricle of male rat 7 days before sacrifice. Adrenalectomy or sham operation was carried out 2 days before sacrifice. Adrenalectomized rats were maintained on normal saline. Each rat received 3.7 μCi/200 gm B.W. of ^{3}H-DA in a volume of 10 μl into right lateral ventricle and was sacrificed at various time after injection. The values are means ± S.E.M.

	Time after Injection	5 minutes		10 minutes		20 minutes		40 minutes	
	OPERATION	Sham	AdX	Sham	AdX	Sham	AdX	Sham	AdX
	Numbers of Animals	6	6	6	6	4	5	5	6
NOREPINEPHRINE	^{3}H-NE formed (ng/gm hypothal)	2.501 ±0.176	2.865 ±0.201	4.352 ±0.345	4.598 ±0.247	5.173 ±0.284	6.174 ±0.475	7.287 ±1.092	6.732 ±0.933
	Content (μg/gm hypothal)	1.826 ±0.075	1.889 ±0.045	1.850 ±0.066	1.776 ±0.052	1.823 ±0.044	1.802 ±0.035	1.874 ±0.074	1.739 ±0.067
	Specific Activity (ng/μg)	1.362 ±0.044	1.512 ±0.090	2.335 ±0.114	2.583 ±0.091	2.833 ±0.106	3.423 ±0.249	3.866 ±0.541	3.828 ±0.437
DOPAMINE	^{3}H-DA remained (ng/gm hypothal)	16.477 ±1.432	16.769 ±1.288	12.201 ±1.018	10.996 ±0.864	7.225 ±0.718	6.554 ±0.729	5.186 ±0.699	4.900 ±0.676
	Content (μg/gm hypothal)	0.741 ±0.045	0.783 ±0.045	0.704 ±0.021	0.682 ±0.011	0.707 ±0.050	0.682 ±0.045	0.631 ±0.022	0.610 ±0.028
	Specific Activity (ng/μg)	22.390 =1.849	21.542 ±1.461	17.498 ±1.742	16.101 ±1.188	10.200 ±0.538	9.666 ±0.948	8.205 ±1.086	7.972 ±1.002
	CONVERSION INDEX (^{3}H-NE/S.A. DA)	0.115 ±0.010	0.133 ±0.004	0.252 ±0.009	0.290 ±0.018	0.510 ±0.033	0.646 ±0.027	0.880 ±0.034	0.862 ±0.087
		P < 0.10		P < 0.09		P < 0.02		P < 0.86	

C. Effect of Adrenalectomy on the Disappearance of ^{3}H-Norepinephrine in the Hypothalamus of the Rat.

The increase in the amount of labeled norepinephrine accumulated in the hypothalamus after adrenalectomy might result from the increased formation or decreased breakdown of norepinephrine, or both. The following experiment was designed to determine whether there was a decreased breakdown of norepinephrine after adrenalectomy. I employed essentially the same technique used by Anton-Tay and Wurtman (1968) in which they studied the effect of gonadectomy on the turnover of norepinephrine in rat brain.

A stainless steel cannula was implanted above the roof of right lateral ventricle of male Sprague-Dawley rats, weighing 160-180 gm, under pentobarbital anesthesia 7 days before sacrifice. Adrenalectomy or sham operation was carried out 2 days before sacrifice. Adrenalectomized rats were maintained on normal saline. Each rat received L-^{3}H-norepinephrine (specific activity 7.3 Ci/mmole), 3 x 10^{5} CPM / 200 gm B.W., in a volume of 17.5 μl into right lateral ventricle through the cannula guide. Earlier investigations had shown that, when ^{3}H-norepinephrine is introduced into the ventricular system in this manner, it mixes bilaterally with the endogenous catecholamine (for reference, see Anton-Tay and Wurtman, 1968). Rats were decapitated at 15 minutes, 2 hours and 20 hours after the injection of labeled norepinephrine. The hypothalamus was dissected out, weighed and homogenized in 5 ml of 0.4 N perchloric acid solution. After the pH of the supernatant was adjusted to 8.4, it was shaken with aluminum oxide to adsorb norepinephrine. The radioactivity remaining unadsorbed in the solution was measured; this represented the portion of O-methylated metabolites of norepinephrine. Labeled norepinephrine in aluminum oxide was eluted with 3 ml of 0.2 N acetic acid, and the radioactivity in the

eluate was determined which represented radioactive norepinephrine taken up and retained by noradrenergic endings in the hypothalamus during the period of study.

TABLE 12-3 Effect of Adrenalectomy on the Disappearance of ^{3}H-Norepinephrine in the Hypothalamus of the Rat.

Each rat received L-^{3}H-norepinephrine, 3 x 10^{5} CPM/200 gm B.W., in a volume of 17.5 µl into the right lateral ventricle through the cannula guide implanted 7 days earlier. Adrenalectomy or sham operation was carried out 2 days prior to sacrifice. Radioactivity was measured in the hypothalamus and expressed in CPM/100 mg hypothalamus. The values are means ± S.E.M.

Time after Injection	Number of Animals	^{3}H-Norepinephrine (CPM/100 mg)	^{3}H-Metabolites (CPM/100 mg)
15 minutes			
Sham	5	11406 ± 1117	13810 ± 1379
AdX	6	10887 ± 1247	11930 ± 1579
2 hours			
Sham	6	4621 ± 226	6531 ± 524
AdX	6	4765 ± 428	6528 ± 499
20 hours			
Sham	6	1223 ± 76	2035 ± 75
AdX	7	975 ± 36*	1872 ± 29@

* $P < 0.01$ vs sham operated.

@ $P < 0.05$ vs sham operated.

Table 12-3 summarizes the results of this experiment. The radioactivity of ^{3}H-norepinephrine and that of its O-methylated metabolites in the hypothalamus of adrenalectomized rats did not change after 15 minutes or 2 hours. However, when the radioactivity was determined 20 hours later, the levels of ^{3}H-norepinephrine and its metabolites decreased by 20 % and 10 %, respectively, in adrenalectomized animals as compared to those in sham operated controls. This indicated that the disappearance of norepinephrine in the hypothalamus was accelerated after adrenalectomy, or in other words, the norepinephrine turnover in the hypothalamus was increased.

DISCUSSION Two days after adrenalectomy, the uptake of tritiated norepinephrine into the hypothalamus after it was introduced into the lateral ventricle did not change, nor was there any difference in the retention of this catecholamine two hours later. Twenty hours after it was injected, however, the amount of labeled norepinephrine remained in the hypothalamus of adrenalectomized rat was less than that of sham operated control. This clearly indicated that there was an increased turnover of norepinephrine in the hypothalamus of the rat two days after adrenalectomy. This finding, together with that of no change in the endogenous content of norepinephrine and that of increased amount of labeled norepinephrine in the hypothalamus after injection of labeled dopamine into lateral ventricle, strongly suggested that the in vivo conversion of dopamine to norepinephrine has indeed been much facilitated two days after adrenalectomy. This observation was unexpected, since it was contradictory to the decreased DBH activity measured by in vitro assay technique.

There are many explanations for this discrepancy. The amount of dopamine-β-hydroxylase present in the hypothalamus may be in excess of the amount required for the optimal conversion of dopamine to norepinephrine

in vivo. Therefore, a decrease of its amount by 10-20 % would not lead to any concomitant decrease in the conversion of dopamine to norepinephrine. Moreover, the regulatory role played by this enzymatic step on the biosynthesis of norepinephrine is probably not simply reflected by the amount of the enzyme present. Rather, it may be other factors that are of importance, such as the pool size of dopamine in noradrenergic endings, the transfer of dopamine into granular vesicles where the conversion takes place, the quantity of endogenous DBH inhibitors and the amounts of required cofactors, in determining the amount of norepinephrine converted from the precursor dopamine. Therefore, the finding that adrenalectomy results in an increased norepinephrine synthesis in the presence of small decrease in DBH content can be explained. This study suggests that the mere measurement of the in vitro activity of an enzyme without reference to its in vivo activity may be misleading. The statement of Pitot and Yatvin (1973) in a review article is pertinent. They pointed out that the assay of an enzyme in vitro in a tissue homogenate is a relatively simple task, whereas the interpretation of the results obtained from such an assay with reference to the metabolic activity of the enzyme in vivo is an entirely different matter. The fact that proteins, and therefore enzymes, are constantly being synthesized and degraded has been well recognized. The fact that DBH is discharged from the nerve endings after each nerve impulse makes the situation more complicated. Before the detailed mechanisms that underly the dynamic turnover of dopamine-β-hydroxylase in the hypothalamus are fully understood, no valid prediction can be made, only based on the measurement of its in vitro activity, as to the in vivo metabolic capacity of this enzyme.

Our finding of increased turnover of norepinephrine in the hypothalamus

of the rat after adrenalectomy is consistent with the results obtained by Javoy et al. (1968) and Fuxe et al. (1973a, 1973b) for the whole brain as well as some other brain areas. The mechanisms which lead to the increase in norepinephrine turnover are obscure. In adrenalectomized rats with or without saline maintenance, the total peripheral resistance was elevated in the first three days after operation (Imms and Neame, 1974), suggesting a generalized increase of sympathetic activity in the periphery. It seems likely that in adrenalectomized rats the increase in norepinephrine turnover in the heart is primarily mediated by a reflex increase in sympathetic nervous activity (Landsberg and Axelrod, 1968; Westfall and Osada, 1969; Daily and Westfall, 1970). Direct influence by hormonal factors, if any, may be of less importance in this instance. It is not clear whether the adrenalectomy-induced increase in norepinephrine turnover in the hypothalamus or whole brain is mediated through the same reflex mechanism in the peripheral sympathetic system. Fuxe et al. (1973a) have suggested that in adrenalectomized rats the increase in norepinephrine turnover is partly mediated via the increase in ACTH secretion, and partly via the loss of circulatory levels of glucocorticoid.

An intriguing question is the effect on the release of corticotropin releasing factor (or hormone) that results from the altered norepinephrine metabolism after adrenalectomy. In order to gain a complete understanding of this subject, much more work must be done.

P A R T III

G E N E R A L D I S C U S S I O N

A N D

C O N C L U S I O N

The work of this dissertation was designed to investigate changes in the activity of dopamine-β-hydroxylase in regions of rat brain under various functional states of the adrenocortical system, so as to elucidate in general whether glucocorticoids have an effect on this norepinephrine synthesizing enzyme in the brain, and in particular whether the feedback action of glucocorticoids on the central nervous system is at this step.

One of the important unsettled questions in hypothalamo-pituitary-adrenal physiology is the site of the glucocorticoid inhibition of ACTH secretion. There is considerable evidence that glucocorticoids inhibit the action of CRF (corticotropin releasing factor or hormone) on the pituitary, and there is some debate concerning an additional feedback of corticosteroids on one or more sites in the central nervous system (for review, see Mangili et al., 1966; Ganong, 1970). For example, direct injection of dexamethasone into the anterior pituitary of the rat in vivo inhibits ACTH release (Russell et al., 1969). Systemic injections of corticosteroids diminish release of ACTH following CRF in animals with parts of the brain damaged or removed in such a way that the source of endogenous CRF is presumably abolished (De Wied, 1964; Dunn and Critchlow, 1969). In such animals corticosteroids can still cause adrenal atrophy, an effect known to follow diminution of ACTH secretion. Furthermore, dexamethasone has been reported to decrease plasma corticosteroid concentrations in hypophysectomized animals with anterior pituitaries transplanted under their renal capsules (Kendall and Allen, 1968). Finally, both incubated pituitary glands and pituitary cells in monolayer culture show corticosteroid inhibition of the ACTH release provoked by various CRFs and ions (Pollock and LaBella, 1966; Fleischer and Vale, 1968; Arimura et al., 1969; Fleischer and Rawls, 1970).

On the other hand, numerous investigators have shown that implants of cortisol and other corticoids in the median eminence inhibited ACTH secretion (for references, see Mangili et al., 1966). In addition, inhibition of ACTH secretion was reported following injection or implantation of corticoids in the midbrain, the septal region, and even the amygdaloid nuclei (for references, see Ganong, 1970). It seems that the nervous system is a site of corticoid feedback. Nevertheless, the implant data must be viewed with caution and other interpretations are possible. Implants in the median eminence region are always subject to the criticism that the steroid is absorbed into the portal vessels and transported to the pituitary. Two laboratories have reported that inhibition of adrenocortical function may result from spread of glucocorticoids implanted in the central nervous system to the pituitary via the cerebrospinal fluid (Kendall et al., 1969; Russell et al., 1969). However, adrenocortical hormones are taken up by brain tissues that include the hypothalamus, the hippocampus, and the septum (McEwen et al., 1969), and it has been shown by autoradiography that at least in the hippocampus, most of the uptake is in neurons (Gerlach and McEwen, 1972). Furthermore, corticosteroids affect brain excitability and morphology (Woodbury, 1958), and one can either increase or decrease neuronal firing rates in the hypothalamus and midbrain by administering steroids (Feldman and Dafny, 1966; Dafny et al., 1973). Therefore, it appears likely that neurons do take up steroids. Finally, two other kinds of evidence suggest a brain site of action for corticoids. First, treatment with glucocorticoids changes hypothalamic CRF content (Chowers et al., 1963; Hedge and Smelik, 1969). Second, systemically administered corticoids have been reported to be more effective in inhibiting increased ACTH secretion produced by stimulation of the amygdala than increased ACTH secretion produced by stimulation of the

hypothalamus (McHugh and Smith, 1967). Thus, it has been established that glucocorticoids feed back at the level of pituitary, and there is additional evidence that they also feed back at the brain level as well.

Dopamine-β-hydroxylase, the final enzyme catalyzing the biosynthesis of norepinephrine, is uniquely localized in the granular vesicles in the cell body and endings of noradrenergic neurons. In peripheral sympathetic nervous system and adrenal medulla, this enzyme is shown to be influenced to certain extent by pituitary-adrenocortical hormones (Weinshilboum and Axelrod, 1970; Gewirtz et al., 1971). Since there are possible differences between peripheral and central neurons in terms of some morphological and functional features, it was of general interest to examine the effect of glucocorticoids on dopamine-β-hydroxylase in the brain. In addition, it was thought that the study might help to elucidate the mechanism by which glucocorticoids exert their feedback effect on the brain to regulate CRF secretion. Although modern pharmacological experiments have provided the evidence that a central noradrenergic mechanism inhibits CRF secretion, they leave unanswered the question of the physiological role of the noradrenergic inhibition. One obvious possibility is that the inhibitory effect of glucocorticoids on ACTH secretion is mediated, at least in part, via the central noradrenergic system. To explore this possibility, I studied the effects of glucocorticoid excess and deficiency upon the activity of dopamine-β-hydroxylase in the brain which is the marker enzyme for noradrenergic neuron. The effect of stress on this enzyme was also studied.

The radioenzymatic assay technique of Molinoff et al. (1971) was adapted and standardized to measure the in vitro activity of dopamine-β-hydroxylase in brain homogenate. The highest activity of DBH was in the hypothalamus, followed by brain stem. I found higher levels of DBH activity in the hypothalamus and brain stem than other workers (Coyle and

Axelrod, 1972; Reis and Molinoff, 1972). The anterior half and ventral half of the hypothalamus have higher DBH activity than the posterior and dorsal halves. Although a clear-cut diurnal fluctuation of ACTH secretion and a minor circadian variation of brain norepinephrine content have been reported, DBH levels in the hypothalamus and the brain stem of the rat appear to be stable throughout the day. Pentobarbital anesthesia did not affect the level of DBH in the brain. Shortly after rats were exposed to ether vapor, there was no change in brain DBH activity. When rats were sacrificed 24 hours later, however, DBH activity in the hypothalamus increased, possibly due to the stress effect of ether in the rat. Injection stress and surgical stress, acute or chronic, did not change DBH activity in the brain. Although immobilization stress acutely decreased DBH content slightly, repeated immobilization seemed to increase DBH in the brain as a consequence of sympathetic and adrenocortical activation.

More direct observation was achieved by studies on the effect of corticosterone excess or deficiency on DBH activity in regions of rat brain. In order to relate the measurement of the in vitro DBH activity to the actual synthetic capacity of the enzyme in living animals, endogenous norepinephrine and dopamine concentrations in brain regions were measured by fluorometric assay. The turnover of catecholamines in noradrenergic neuronal elements was assessed by estimating the appearance or disappearance of radioactive norepinephrine in the hypothalamus after labeled dopamine or norepinephrine was introduced into the cerebral ventricles.

Four hours after large dose of corticosterone, DBH activity in the hypothalamus increased significantly by 7 %, whereas that in the brain stem or hippocampus remained unchanged, indicating that the acute effect of glucocorticoids on DBH in the brain is region-specific. No parallel increase in the static concentration of norepinephrine or dopamine could

be obtained, nor was there any change in the accumulation of radioactive norepinephrine in the hypothalamus 40 minutes after ^{3}H-dopamine was introduced into the third ventricle. When the rat received treatment with corticosterone daily for 5 days, DBH activity in the hypothalamus, brain stem and hippocampus all increased by 15-20 %. The norepinephrine and dopamine concentrations in the hypothalamus decreased, whereas the norepinephrine and dopamine concentrations in the brain stem did not show any change.

A 10-20 % decrease in DBH activity in both the hypothalamus and brain stem was observed one to two days after adrenalectomy; this decrease was not restored by moderate dose of corticosterone replacement, nor was this decrease attributable to sodium deficiency or lack of neural connections between adrenal gland and the central nervous system. In addition, this decrease in DBH activity was not accompanied by any change in norepinephrine or dopamine content. Five days after adrenalectomy DBH activity in the hypothalamus returned to normal, while that in brain stem remained low. The conversion of ^{3}H-dopamine to ^{3}H-norepinephrine in the hypothalamus of living animal was accelerated two days after adrenalectomy when there was a decrease in DBH activity measured by the *in vitro* technique.

The data accumulated indicate that adrenalectomy decreases DBH activity in the hypothalamus and brain stem, that large dose of corticosterone increases it, and that physiological dose of glucocorticoid fails to restore the decreased DBH activity in the brain of adrenalectomized rat. They indicate that the adrenal gland regulates DBH activity in the hypothalamus and brain stem of the rat. However, the effect is not mediated by glucocorticoids alone; some other factor in the adrenal gland, such as adrenal medullary hormones and other secretory products of the adrenal

cortex, may be responsible for this regulatory mechanism.

The changes in DBH activity after glucocorticoid deficiency or excess are not of great magnitude. Minor changes in DBH activity are not accompanied by parallel changes of catecholamine content or norepinephrine formation. In contrast, the decrease in hypothalamic DBH activity in adrenalectomized rats is associated with an increase in the dynamic turnover of norepinephrine, manifested by both the increased formation and increased disposition of the neurotransmitter. These findings clearly illustrate that a change in the activity of hypothalamic DBH measured by the in vitro assay is not necessary associated with a parallel change in the turnover of norepinephrine in the living animal. This seems to suggest that dopamine-β-hydroxylase in the hypothalamus is not rate-limiting for the biosynthesis of norepinephrine. The static content of DBH measured by the in vitro assay can not represent its in vivo synthetic capacity because of many special features of this enzyme and the reaction it catalyzes. It might be these factors that influence the amount of norepinephrine newly synthesized from its immediate precursor. In the first place, the enzyme molecules and substrates of this pathway do not have free access to one another since dopamine-β-hydroxylase is sequestered in noradrenergic vesicles. Thus, the amount of dopamine entering the vesicles may determine the amount of norepinephrine formed. In the second place, the catecholamine biosynthetic pathway is highly branched. Some of the dopamine is taken up into vesicles and converted to norepinephrine, and some is deaminated by the intraneuronal monoamine oxidase. A competition therefore exists between deamination and uptake/beta-hydroxylation. Any situation which changes the capacity of the neuron to catabolyze dopamine would change the formation of norepinephrine as the consequence. Thirdly, the

availability of the essential cofactors (ascorbate, oxygen, dicarboxylic acids, etc.) may also affect the reaction rate. The last potential mechanism for regulating DBH activity in vivo involves the endogenous inhibitors of this enzyme. It is very possible that DBH inhibitors are playing a significant physiological role in regulating DBH activity because it has been shown that they indeed have access to the enzyme. A change in the amount of endogenous inhibitors provoked by physiological manipulation will change the in vivo synthetic capacity of DBH even though there is no detectable change in the static content of DBH measured by the in vitro assay. Therefore, the observed discrepancy between the in vitro and in vivo DBH activity in the hypothalamus of adrenalectomized rat can be explained.

Several interesting questions are raised or left unanswered after this dissertation work. First of all, the mechanism for the discharge of norepinephrine, dopamine-β-hydroxylase and other soluble contents of the noradrenergic vesicles in the brain after nerve impulse has not been uncovered. It is only speculated that the process follows that of exocytosis in the adrenal medulla and peripheral sympathetic nerve endings. Nor have the fate of discharged DBH and the mechanism of DBH supply from the soma to nerve endings been studied. A possible way to attack the problem is to measure the amount of DBH in the cerebrospinal fluid and venous blood from the brain after electrical stimulation of noradrenergic pathways in the brain. This may provide clue to what happens to the secreted DBH in the brain. It has been shown that less than 10 % of DBH in the hypothalamus is in soluble form which may be discharged after nerve impulse (Belmar et al., 1974). Thus, the DBH activity measured in homogenates of brain tissue can be a reasonable measure of the neuronal content of this enzyme. Since the fluxes of DBH in and out of the nerve ending determine its

content in that neuronal structure, the measurement of the turnover of this enzyme appears to be more meaningful than the measurement of its static tissue content. Yet no method for studying this is currently available. In addition, it should be kept in mind that the measurement of DBH content in the brain by the in vitro radioenzymatic assay is highly artificial, and may not coincide well with the real in vivo activity of the enzyme.

Equally important is the consideration of the influence on the in vivo activity of DBH by endogenous inhibitors. The assay for the inhibitors of DBH has been established (foldes et al., 1972), and their properties have been studied. In current experiments Molinoff and associates have started to investigate the possibility that DBH inhibitors are playing a significant physiological role in regulating DBH activity (Molinoff and Orcutt, 1973). The concentration of inhibitors in the nerve may change in response to exogenous stimuli. Therefore, the study of the change in endogenous inhibitors after manipulation of adrenocortical system may be interesting as well. Although the measurement of the changes in enzyme content in various functional states of the endocrine systems constitutes an important area in the field of molecular biology in the regulation of enzyme protein, this turns out not to be a satisfactory approach toward the mechanism of hormonal feedback on central neurons. Studies of the dynamic metabolism of norepinephrine in specified regions of the brain appear to be more appropriate.

One question left unanswered by this research is the mechanism and the site in the brain of corticosteroid feedback control on the secretion of corticotropin releasing factor. On the basis of my observations, it is difficult to conclude whether the site of glucocorticoid feedback on

the central nervous system is in the noradrenergic neurons, or more specifically, whether it is at this enzymatic step. More future work is required to settle this important physiological problem. In addition, all the findings in such studies must be interpreted with caution. Being able to demonstrate changes in the metabolism of noradrenergic neurons in the hypothalamus or other regions of the brain does not provide conclusive evidence that the secretion of CRF will be affected as a consequence to that change.

In the studies on the biochemical properties of partially purified dopamine-β-hydroxylase from beef adrenal gland, it was demonstrated that ascorbic acid is essential for the in vitro DBH activity, that it can be replaced by a NADH-phenazine methosulfate system which generates superoxide anions, thus suggesting that the role of ascorbate is probably in the generation of superoxide free radicals which are responsible for the activation of the enzyme. It was also demonstrated that hydralazine, an antihypertensive drug, may inhibit DBH activity in vitro by chelating cupric ions which are required for the neutralization of endogenous DBH inhibitors present in the crude enzyme preparations.

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