A STUDY OF SEVERAL ASPECTS OF THE ENZYME TYROSINE HYDROXYLASE

by

Sheila M. Gibson

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Department of PSYCHIATRY

The University of British Columbia
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ABSTRACT

Interest in brain catecholamines has grown considerably in the last few years in view of their possible role as neurotransmitters.

This investigation deals primarily with the enzyme tyrosine hydroxylase which is thought to be the rate limiting step in catecholamine synthesis.

Using tyrosine hydroxylase measurements and catecholamine depletion techniques, attempts were made to determine the site of increased synthesis of catecholamines in animals exposed to cold. Brain, heart and spleen do not appear to be the organs involved in this change. Adrenals may be of significance but the results were suggestive rather than conclusive.

Tyrosine hydroxylase distribution in brain was determined in various regions of rat, rabbit and cat brain, and activity was shown to be highest in the caudate, septal area, nucleus accombens, and anterior perforating substance, with much lower activities in other regions such as hippocampus, amygdala, hypothalamus, thalamus, cortex, cerebellum and brain stem.

Using these distribution studies as indications of normal tyrosine hydroxylase activity in areas of rat brain, and electrolytic lesion techniques, studies were carried out to determine noradrenergic and dopanergic pathways in brain. Catecholamine fibers from their origin in the midbrain were traced in the midbrain and diencephalon to the caudate and septal area, and the relative positions of each group of fibers determined along their course.

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INTRODUCTION

Recently it has become evident that the catecholamines (CA) have a significant role in brain function. Therefore, a study of these compounds should aid in elucidating some of the biochemical mechanisms of the central nervous system (CNS).

1. Catecholamines

a) Chemistry

The basic structure of the CA is a dihydroxy aromatic ring (catechol moiety) with a two carbon side chain containing an amine group. Variations within the CA arise from substitutions on the β carbon and/or the amine group. The important members of the CA family, noradrenaline (NA), dopamine (DA) and adrenaline, are shown in Fig. 1.

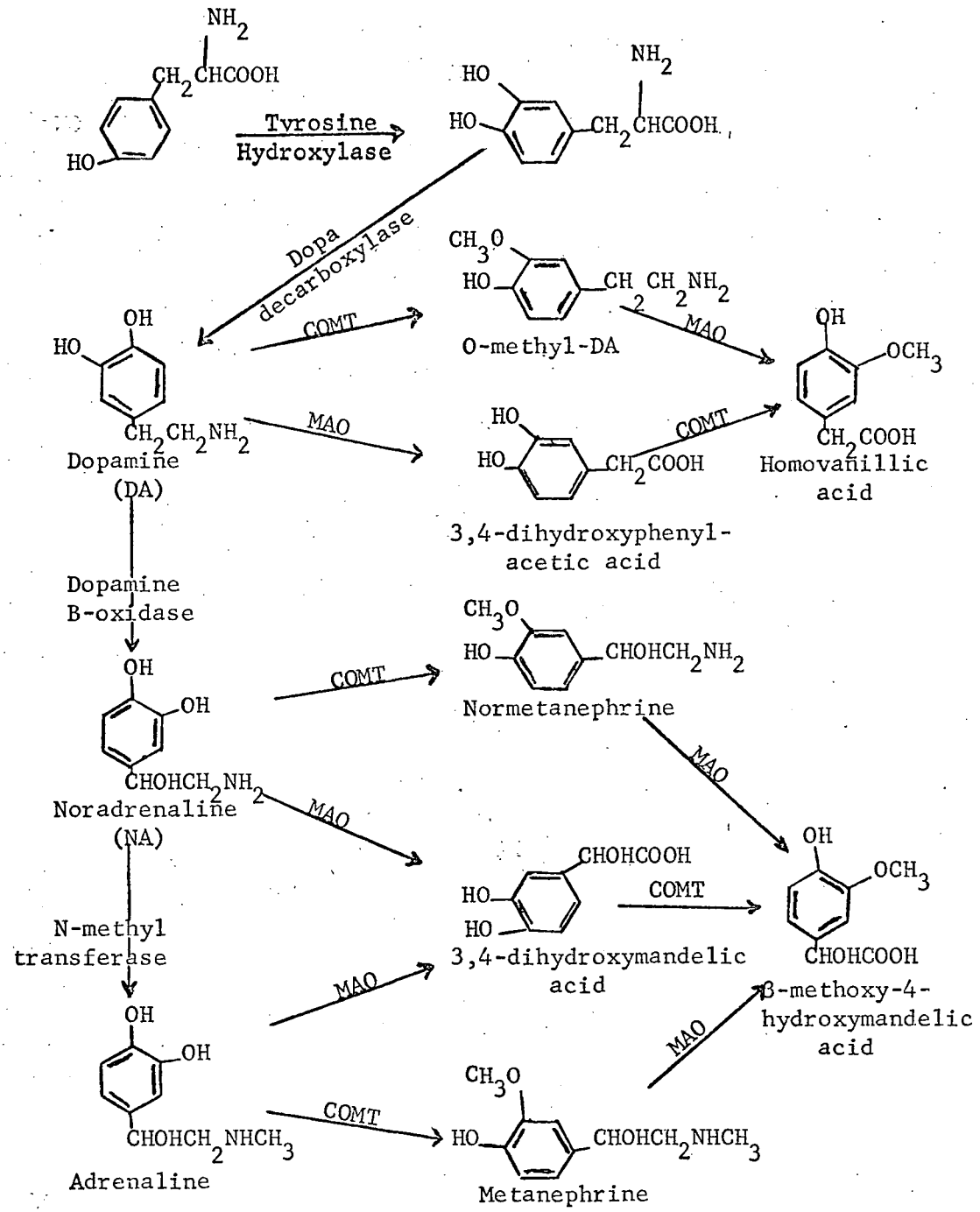
b) Metabolism

The steps in the *in vivo* synthesis and catabolism of the CA have been worked out. The sequence and necessary enzymes are presented in Fig. 1. It is worth noting that NA and DA have significant physiological activity as well as being precursors in adrenaline synthesis. The CAs are inactivated *in vivo* not only by O-methylation and oxidation as shown in Fig. 1, but by re-uptake into tissue and by diffusion away from the site of activity. The CA and metabolites are eventually excreted via the kidney and can be detected in the urine.

c) Location

The CAs are found in various organs of the body: e.g. brain and adrenals as well as in all sympathetically innervated tissue.

In the adrenals NA and adrenaline are contained in vesicles within chromaffin cells, and are released on stimulation of the splanchnic nerve. DA is also present but probably only as a precursor (1). In the rest of the periphery, NA is contained in the nerve endings, both in vesicles, that are depleted on nerve stimulation, and in the cytoplasm (see Diag. 1).



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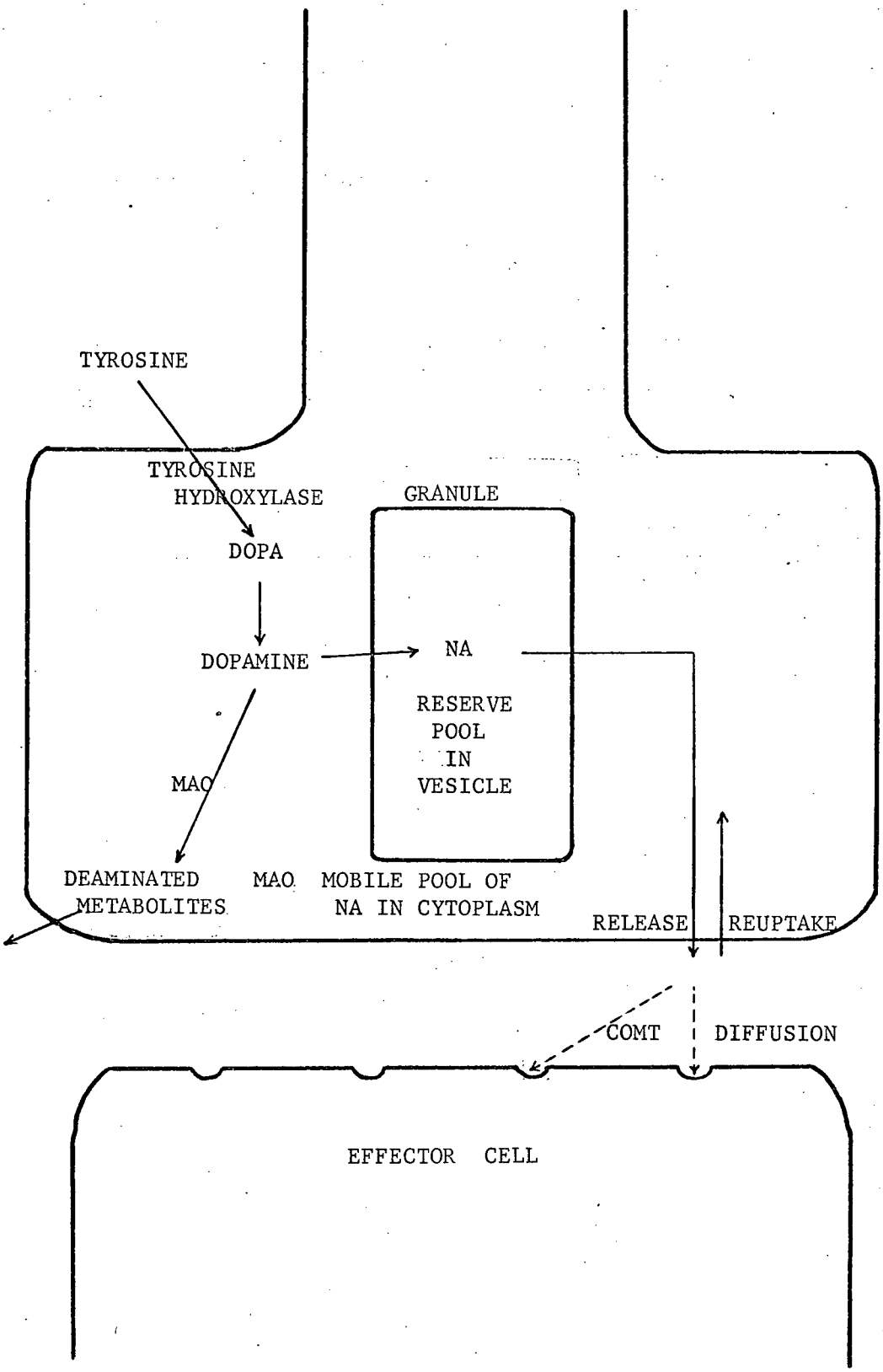
COMT	catechol-O-methyltransferase
MAO	monamine oxidase

Fig. 1: Metabolism of Catecholamine



Diag. 1: Subcellular location of noradrenaline in the nerve endings of the sympathetic nervous system.

NA and DA are present in brain at approximately 1% of the concentration found in adrenals. Here DA appears to have some role in addition to that as a precursor for NA synthesis.

As shown in Table 1. both chemical (2,3,4,5) and histochemical (6,7) analyses indicate an uneven distribution of NA and DA in the brain. DA is concentrated in the striatum, NA in the hypothalamus, but both are found in significant concentrations throughout the limbic system. It has also been found by radioactive tracer work that NA and DA injected into the brain are taken up preferentially into certain areas (8) with a distribution similar to that of the endogenous amine. For example, if radioactive NA is injected into rat brain, the concentration after one hour in the hypothalamus is 10 times that of the cortex. Also indicated in Table 1 are the differences in rate of *in vivo* synthesis of CA in the various regions of brain (9). By injection of the radioactive precursor tyrosine it has been demonstrated that the type of CA synthesis varies throughout the brain; the major product of CA synthesis is DA in the striatum and NA in the hypothalamus (10).

As in the periphery, NA in the brain has been detected in presynaptic vesicles of nerve endings (11,12). Histochemical studies also indicate that DA is located primarily in nerve endings (13).

d) Function

The functions of CA in the periphery are fairly well established. When an animal is placed under stress there is an increased neuronal activity in the sympathetic nervous system, resulting in NA and adrenaline release from the adrenals and a discharge of NA from sympathetic neurons. NA causes vasoconstriction in the gastrointestinal tract, skin and kidney; inhibition of intestinal smooth muscle contraction, pupil dilation and increase in heart rate and force of contraction. Adrenaline has similar effects on the pupils, smooth muscle and heart and in addition, causes vasodilation in muscle, dilation of bronchi, mobilization of fatty acids and increased glucose metabolism.

TABLE 1

SUMMARY OF DISTRIBUTION DATA FOR MAJOR AREAS OF BRAIN

Area	Biochemical* NA	Biochemical* DA	Histochemical+ Description of Monoamine Terminals	Turnover0 NA	Turnover0 DA
	γ/gm	γ/gm	intensity and type	mμg/gm/hr	
Caudate	0.3 - 0.6	3.1 - 7.5	strong diffuse fluorescence of DA		900 - 2400
Septum	0.7 - 1.5	1.6	low to medium of NA; dotted strong intensity of DA		
Hypothalamus	0.7	1.8 - 3.6	low to very strong of NA (in different nuclei)	234	
Amygdala	0.2 - 0.3	0.2	medium of NA		
Hippocampus	0.1 - 0.2	0.1 - 0.2	low to high of NA	33	
Thalamus	0.2 - 0.4	0.5	predominantly low of NA - two very high nuclei		
Midbrain	0.3 - 0.5	0.2	wide variation depending on nuclei		
Pons-Medulla Oblongata	0.1 - 0.4	0.1 - 0.3	wide variation	90	
Cerebellum	0.1 - 0.2	0.03- 0.1	low - mostly NA	42	
Cortex	0.1 - 0.3	0.1 - 0.3		36	

* summary of data from references 2,3,4 and 5 + from reference 7 0 from reference 9

These physiological changes enable the animal to meet many life threatening situations with a "fight or flight" response.

Throughout the periphery NA, but not adrenaline, acts as a neurotransmitter in postganglionic neurons of the sympathetic nervous system.

Although the evidence is not as conclusive, it is also thought that NA and DA are neurotransmitters in the CNS (14,15). The indications for this role are:

1) NA and DA are present in brain in the appropriate concentrations (16),

2) enzymes for their formation and destruction are present (17,2,18,19),

3) agents that act as agonists and antagonists at noradrenergic synapses in the periphery also affect the central nervous system (15),

4) release of CA is observed on stimulation of certain nerve tracts (20,21),

5) the CAs are located in nerve endings (11,12,13) and

6) there is some indication that application of CA to synapses affect post synaptic potentials (22).

2. Tyrosine Hydroxylase

a) As a control of CA synthesis

The enzymes active in the biosynthesis of CA, that is tyrosine hydroxylase, dopa decarboxylase, dopamine β oxidase and N-methyl transferase have been studied a great deal in adrenal medulla (23,24,25,26). In brain, only tyrosine hydroxylase (27) and dopa decarboxylase (2) have been worked on extensively, (N-methyl transferase is not present to any extent). From these studies tyrosine hydroxylase appears to be the rate limiting enzyme, as would be expected since it is the first enzyme in the biosynthetic route. The other enzymes in the sequence have activities in the order of 10,000 μmoles/gm/hr whereas tyrosine hydroxylase has an activity of 4-100 μmoles/gm/hr. The K_m for

overall conversion of tyrosine to CAs is 1×10^{-5}, the approximate K_m for tyrosine hydroxylase (28). Inhibitors of tyrosine hydroxylase are more effective than inhibitors of dopamine β oxidase and dopa decarboxylase in reducing CA synthesis; the reduction in NA is proportional to the degree of tyrosine hydroxylase inhibition(29). From these findings Udenfriend (28) proposed that the conversion of tyrosine to Dopa is the rate limiting step in CA synthesis, because the amount of enzyme present in the tissue is limiting.

According to the in vivo turnover studies of Glowinski (30,31,32) CA concentrations do not necessarily reflect CA turnover. Glowinski found, for example, that the cerebellum, although it is low in CA, has one of the highest turnover rates. Such in vivo studies of CA turnover involve long and cumbersome procedures and give inconsistent results. Therefore measurement of tyrosine hydroxylase in vitro activity may be more helpful in obtaining an accurate picture of CA turnover in vivo.

b) Characteristics

Although the presence of tyrosine hydroxylase in brain had been established by in vivo work before (10), it had not been possible to detect in vitro activity. Low enzymatic activity and high tyrosine concentrations in tissue meant that [illegible] ^{14}C-tyrosine of high isotopic enrichment was required. Udenfriend (33,34) was the first to show an enzymatic conversion of L-tyrosine to Dopa. Using purified beef adrenal, the conditions found for maximum in vitro activity were acetate buffer, pH 6.0, in air and in the presence of the cofactor, 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine ($DMPH_4$) in mercaptoethanol. Work by Udenfriend and Kaufman (35) indicate that maximum activity of tyrosine hydroxylase occurs in the presence of pteridine cofactor which apparently increases the affinity of the enzyme for tyrosine. The proposed mechanism of hydroxylation involves the reduction of enzyme by $DMPH_4$ enabling the enzyme to aerobically oxidize tyrosine to Dopa.

Work in this laboratory has confirmed the characteristics of tyrosine hydroxylase in crude beef adrenal homogenate. But in crude beef, rat, rabbit and cat brain homogenates, different results were obtained (27,36). Maximum in vitro activity was found to be with PO_4 buffer pH 6.2, in air; $DMPH_4$ does not enhance activity. The K_m value in brain ranged from 0.5×10^{-5} to 1×10^{-5} for the above tissues as compared to 2×10^{-5} for adrenal.

c) Location

i) Subcellular localization

Using sucrose gradient techniques, tyrosine hydroxylase activity has been located in the nerve endings (37). However the distribution within the synaptosome has not been precisely established. Originally tyrosine hydroxylase was thought to be a soluble enzyme in adrenal, since most of the activity was found to be in the supernatant after centrifugation at 105,000 g (33). More recently it has been reported to be particle bound (38). In splanchnic nerve it has been demonstrated that tyrosine hydroxylase and dopa decarboxylase are in the cytoplasma (39,40). Dopamine β oxidase is a particle bound enzyme possibly part of, or within, the vesicles storing NA. In brain two separate groups of workers (41,37) have shown that tyrosine hydroxylase is particle bound, but its exact location is not known.

ii) Anatomical location

Considering gross anatomy, tyrosine hydroxylase is most active in adrenals and brain with some indication that it is present in heart and spleen (34). Tyrosine hydroxylase distribution in various brain areas has not been thoroughly investigated.

d) Inhibitors

Two main classes of tyrosine hydroxylase inhibitors have been found, catechols and aromatic amino acids (41,42,43,44). According to kinetic studies of Udenfriend, Dopa and the catechols are competitive inhibitors of $DMPH_4$ and non-competitive inhibitors of tyrosine, while the amino acids are competitive with tyrosine for sites on the enzyme. The CAs themselves can inhibit tyrosine

hydroxylase and possibly act as feedback regulators of synthesis. Other potent catechol-type inhibitors are α-methyl Dopa, epinine and methylaminoacetocatechol. Examples of potent amino acid inhibitors are α-methyl-p-tyrosine, 3-iodo-tyrosine and halo-tryptophans. These compounds have been useful for studying turn-over rates of CAs in vivo (32) and behavioral effects of altered CA levels (45,46).

3. Dopanergic and Noradrenergic Pathways in Brain

For many years anatomists have used nerve degeneration resulting from lesions to study neuronal pathways in the central nervous system and periphery. Biochemical changes can also be observed in degenerated axons. Cutting of the post-ganglionic neuron in the sympathetic nerve results in decreases in NA content of innervated organs (47,48). These principles can be applied to the central nervous system: using stereotaxic apparatus, precise electrolytic lesions can be placed in brain, and subsequent measurement of changes in CA concentrations. can be used to trace dopanergic and noradrenergic pathways

The most extensive mapping of such paths has been done by a group of Swedish (6,7,49,50,51) workers using histochemical techniques on rats. They describe DA-containing neurons originating in the midbrain nuclei, i.e. zona compacta of the substantia nigra, ventrolateral portion of the reticular formation, and cranial half of nucleus interpeduncularis. These cell bodies send axons caudally to the striatum, tuberculum olfactorium and nucleus accombens on the ipsilateral side. The cell bodies of NA neurons are in the pons and medulla oblongata. Most of the noradrenergic fibres descend to the spinal cord. However, fibres from cells in the ventrolateral part of the reticular formation send axons rostrally through the tegmentum of the midbrain and medial forebrain bundle (MFB) to the hypothalamus, pre-optic area, septal area, amygdala, hippocampus and cingulate gyrus. The results of these experiments are summarized in Fig. 2.

Using biochemical techniques other workers have confirmed some of these results. Poirier and Sourkes (53,54) using cats and monkeys, showed decreases in DA of the striatum with lesions and chromatolysis of nerve cell bodies in the substantia nigra and parabracheolis pigmentosis. These decreases could not be reversed with MAO inhibitors. Goldstein (54,55,56) also working on the nigrostriatal tract, demonstrated that lesions of the ventromedial tegmentum of the midbrain caused decreases in DA concentrations, decrease in synthesis of DA from ^{14}C-tyrosine and decreases in uptake of radioactive DA in the ipsilateral caudate and putamen. Moore and Heller (57,58,59) have reported decreases in NA with lesions of the MFB within the lateral hypothalamus; areas showing decrease include the septum, striatum, amygdala and hippocampus.

Anatomical studies have been generally unsuccessful in demonstrating these CA-containing tracts (60,61,62). Lesions of the substantia nigra indicate efferents to the red nucleus, superior colliculi, thalamus and globus pallidus. Nauta (63) claims there are ascending fibres from the midbrain to caudate and putamen via the internal capsule. He (64) and others (65,66) also describe fibers in the MFB to the amygdala, thalamus, septal nuclei, diagonal band nuclei, hippocampus and hypothalamus.

4. Possible Role of Catecholamines in Brain Function

Since the CAs are probably neurotransmitters in the central nervous system; it would be assumed they influence specific functions in the brain. Some work has been done in trying to relate CA and brain function.

a) Emotion and Behavior

Papez (67) was the first to put the nebulous term emotion into something "concrete" by describing an "emotional circuit," consisting of hypothalamus, thalamus, cingulate gyrus and hippocampus. This circuit is now thought to involve most of the limbic system (14). The hippocampus and amygdala appear to be the central turnover points where a stimulus is transduced into a precise somatic

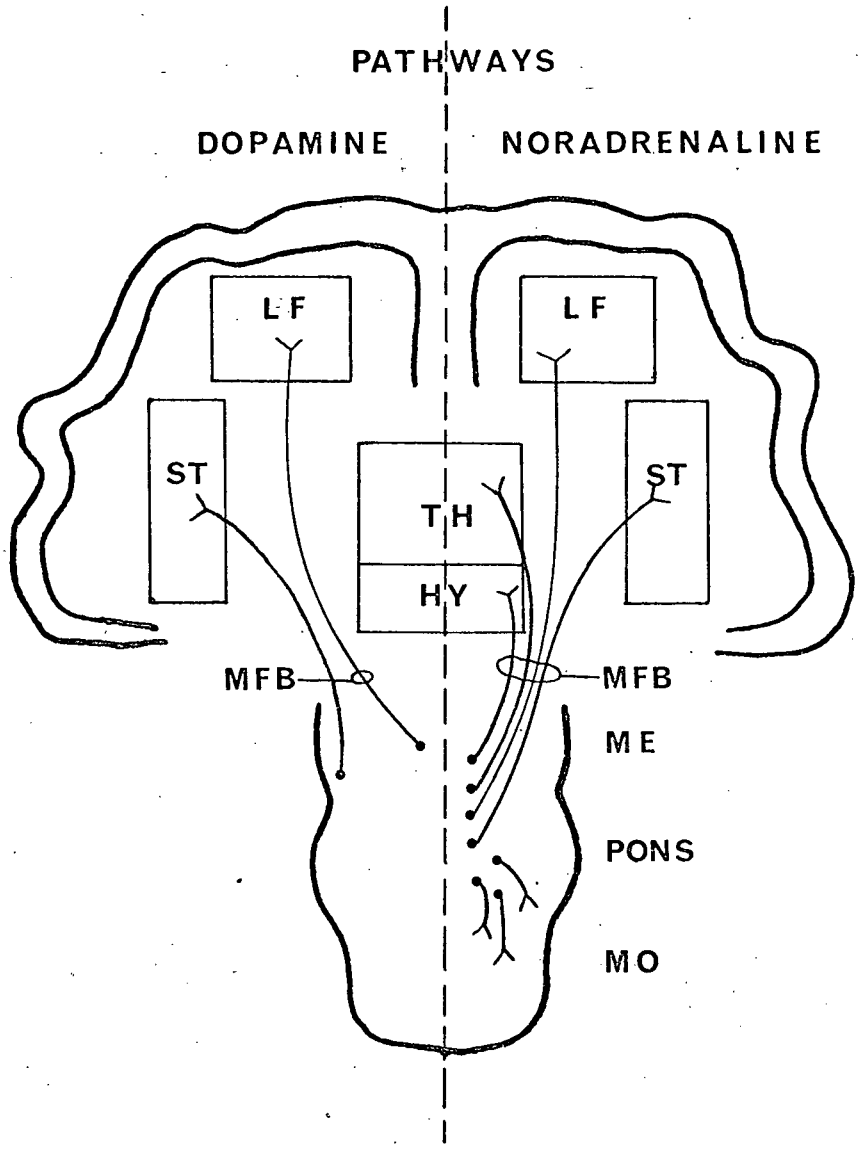


Fig. 2: Summary of noradrenergic and dopaminergic tracts in brain

LF = limbic forebrain MFB = medial forebrain bundle

ST = striatum ME = mesencephalon

TH = thalamus MO = medulla oblongata

HY = hypothalamus

From reference 15

emotional responses which is expressed via the other components of the circuit. The septal area may function as a type of activating system for the hippocampus. It has further been suggested that the hippocampus-amygdala complex is susceptible to breakdown. The result of such a disruption could be mental illness. As already indicated, these areas are relatively high in CAs. It has been hypothesized that they may play some role as neurotransmitters in the "emotion circuit." To substantiate this theory it has been found that CA agonists and antagonists can be used in the treatment of mental illness. In general, drugs that antagonize CA activity are mood depressants and those that act as agonists are mood elevators (15). The hypothesis (15,68) is that mood changes are caused by activation or depression of synapses in the limbic lobe. Since the method of mood development is not known, and these agents can have several effects on CA and possibly other systems, the exact mechanism of action of these drugs in changing mood is obscure.

Numerous animal experiments have been done in an attempt to relate emotion, behavior and CA. Examples of these are:

1) stimulation of the amygdala in cats produces sham rage along with a decrease in NA in the brain (69),

2) brainstem transections that evoke defense reactions cause decrease in NA in brain (70),

3) self stimulation of pleasure centres of the hypothalamus is inhibited by decreasing NA concentration and enhanced by the NA agonist, metanephrine, suggesting that NA may be the neurotransmitter in this pleasure system (46), and

4) animals in groups (social stress) have more CA and serotonin in the brain, tend to be less aggressive and responsive to stimuli than do isolated animals (71,72,73).

The suggestion is that in the grouped animals with excess transmitters the receptors are desensitized and therefore less responsive. This could be a sort of emotional safety mechanism. Other similar studies (74,75,45) have been done but no conclusive results relating CA, behavior and emotion have been obtained.

b) Memory and Learning

As well as being part of the "emotion circuit" the hippocampus is also important in memory (77). Whether the CAs present there or in other regions of the brain are significant in memory is not known. Wada and McGeer (78) showed that an increase in DA and NA enhanced learning while low levels inhibited learning. Others (79) have reported that decreases in cerebral CA and serotonin concentrations enhanced learning. With respect to the already learned response, it has been reported (80,81,82) that antagonists of NA activity blocked conditioned behavior response.

c) Sleep

Sleep is a poorly understood phenomenon in which CA may play an important role. During a sleep period animals alternate between light sleep and paradoxical sleep. Jouvet (83,84) has proposed that NA from the nuclei locus caeruleus in the tegmentum of the pons acts as mediator in the initiation of paradoxical sleep. Narcolepsy, a pathological condition which causes a sudden onset of sleep, can be controlled by the NA agonist, amphetamine (85); and NA applied to the reticular formation can cause an arousal response (86). These latter observations would indicate their may be a relationship between CA and sleep.

d) Temperature Regulation

By lesion and stimulation studies it has been established that the hypothalamus is prominently concerned in body temperature control. Feldbury and Meyer (87) and others (88) have postulated that temperature regulation takes place by a balance of the hyperthermic effects of serotonin and of NA. It has also been fairly well established by Leduc (89) that the CA, whose excretion is increased when animals are placed in the cold, are important in adaption to cold. It has not been determined if this CA influence on cold acclimitization is peripheral only or whether there is also a CNS component.

e) Basal Ganglia and Parkinsonism

Through studies of DA metabolism and dopanergic pathways the

function and mechanism of action of the basal ganglia are being elucidated. In patients suffering from Parkinsonism, a disease apparently caused by malfunction of the basal ganglia and some of its connections (e.g. substantia nigra), urinary secretions of DA and its metabolites are decreased (90). After death analysis of the basal ganglia of these patients show a decrease in DA content (91). Agents such as reserpine that (among other actions) deplete DA from the basal ganglia cause Parkinsonism - like symptoms (92), and administration of large doses of Dopa have been reported to alleviate the symptoms of Parkinsonism in some cases (93). Lesions of the nigrostriatal tract in monkeys in some cases can produce tremor and rigidity (94). From there and other findings it has been suggested that DA acts as an inhibitory neurotransmitter in the basal ganglia and that Parkinsonism may in part be a result of inadequate inhibition in the extra-pyramidal motor system as a result of lack of neurotransmitter DA (95,96).

In all the aspects of brain function mentioned there are hints of relationships between CA and brain mechanisms, but no definite working hypothesis can be established. Much more work must be done on the CA in brain.

5. Aims of Thesis

The purpose of this investigation is to study several aspects of the significant enzyme in CA synthesis, tyrosine hydroxylase. There will be three main considerations:

1) The effects on tyrosine hydroxylase activity in brain of altering CA levels in the body will be determined. These alterations are brought about by placing the animal in the cold, and by artificially changing the levels with drugs (substances that deplete CA stores and MAO inhibitors that increase CA concentrations).

2) An extensive study will be made of tyrosine hydroxylase activity in various regions of brain in several animals.

3) On the basis of these normal distribution studies, and

the fact that tyrosine hydroxylase is contained in nerve endings, the third aim of this study wil be the investigation of the anatomy of the noradrenergic and dopanergic pathways in brain. Le sion techniques coupled with enzyme measurements will be used.

METHODS AND MATERIALS

a) Tyrosine Hydroxylase Analysis

i) Procedure

Tissue was homogenized in 4 - 9 volumes of sucrose. The incubation mixture consisted of 0.1 ml 0.28 M PO_4 buffer pH 6.2, 0.1 ml of homogenate, and 0.1 ml of a solution of uniformly labelled L-tyrosine-^{14}C (150,000 cpm, sp. act. 360-375 mc/mmoles) which was 3 x 10^{-3}M in N-methyl-N-3-hydroxyphenylhydrazine (NSD-1034) in distilled water. When $DMPH_4$ was used, 0.07 ml of 0.40 M PO_4 buffer and 0.03 ml of 2 x 10^{-4} $DMPH_4$ in 0.02M 2-mercaptoethanol were added instead of the 0.1 ml of 0.28M buffer. The incubation was carried out in air at 37°C for 30 minutes and stopped with the addition of 2 ml of a 1:1 mixture of 0.2M HAc and 0.4M $HClO_4$ containing 0.1 ug/ml of Dopa, NA and DA. Blanks were run at the same time using tissue that had been heated at 80 - 90°C for 10 - 15 minutes. Duplicates were run for each sample. In most cases the acidified incubates were frozen before isolation of the catechols.

Isolation of the radioactive catechols formed was on aluminum oxide. The incubation mixture was thawed, centrifuged and the supernatant poured into a 20 ml beaker containing 1 ml of 0.2M EDTA. The precipitate was washed with 3 ml of 0.32M PO_4, recentrifuged and the supernatant pooled with the other. Each sample was taken to pH 8.8 - 9.2 with NaOH and 300 mg of alumina was added to the beaker. The mixture was stirred for 4 - 5 minutes and then washed into a small glass column (4 mm diameter) stoppered with glass wool. Gentle suction was used to draw the liquid through the column. The column was further washed with 2 lots of water (ca 10 ml each). The catechols were eluted from the column with 2 ml of 0.5N HAc into a small vial. Ten ml of Bray's solution were added and the vials were counted in a liquid-scintillation spectrophotometer. The whole isolation procedure took approximately 10 minutes per sample.

ii) Rational for Procedure

Conditions for incubation and isolation had previously been worked out (27) and diagrams presented in this thesis are confirmation

of these.

The volumes of sucrose were chosen to give 10 - 20 mg of tissue per incubation. The amount used in any given incubation depended on the amount of tissue available and the activity of the tissue. As shown in Fig. 3 the activity is linear with mg of tissue used in this range. Using sucrose of molarity 0.2 - 0.3 it has been shown (27) that 0.28 gives the best conversion. As shown in Fig. 4 the maximum pH for the reaction is 6.0 - 6.4. Therefore the intermediate pH 6.2 was chosen. As previously demonstrated (27) PO_4 buffer gave maximal results for brain tissue. The L-tyrosine-^{14}C from the manufacturer was diluted so that each incubation mixture contained 1/12 μc (150,000 cpm). For whole rat brain this resulted in approximately 2000 cpm of catechols formed with a blank of 150 - 200 cpm. Therefore, this amount appeared to be a good compromise between reasonable conversion, low blank and cost. NSD-1034 is a potent Dopa decarboxylase inhibitor (97), and blocks the reaction is at the Dopa stage. If NA and DA were formed they would further be metabolized by O-methyl transferase and monoamine oxidase. This would make isolation more difficult. Addition of cold catechols before isolation improves the recovery. Incubation is linear with time up to 45 minutes (Fig. 5). Thirty minutes was chosen for convenience.

It has been reported that $DMPH_4$ is necessary for maximum tyrosine hydroxylase activity in purified beef adrenal (23). Incubations carried out with and without $DMPH_4$ are presented in Table 2. There was no significant difference in tyrosine hydroxylase activity of brain homogenates with and without cofactor. There was a 10 fold increase in activity with cat adrenals, indicating that the $DMPH_4$ had not been decomposed. Because of these results the cofactor was not used routinely in the brain tyrosine hydroxylase homogenates.

Oxygen is necessary for the reaction but 20% saturates the enzyme (23). The incubations are therefore carried out in air.

Alumina has proven to be one of the most satisfactory and simplest methods for isolation catechols (98). Uptake on the column is

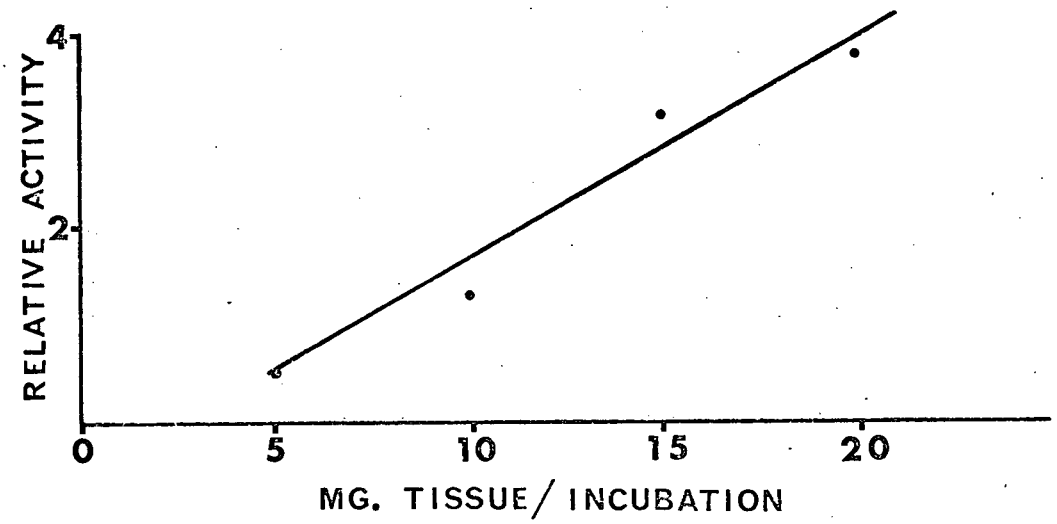


Fig. 3: Relationship between amount of brain tissue used in incubation and tyrosine hydroxylase activity

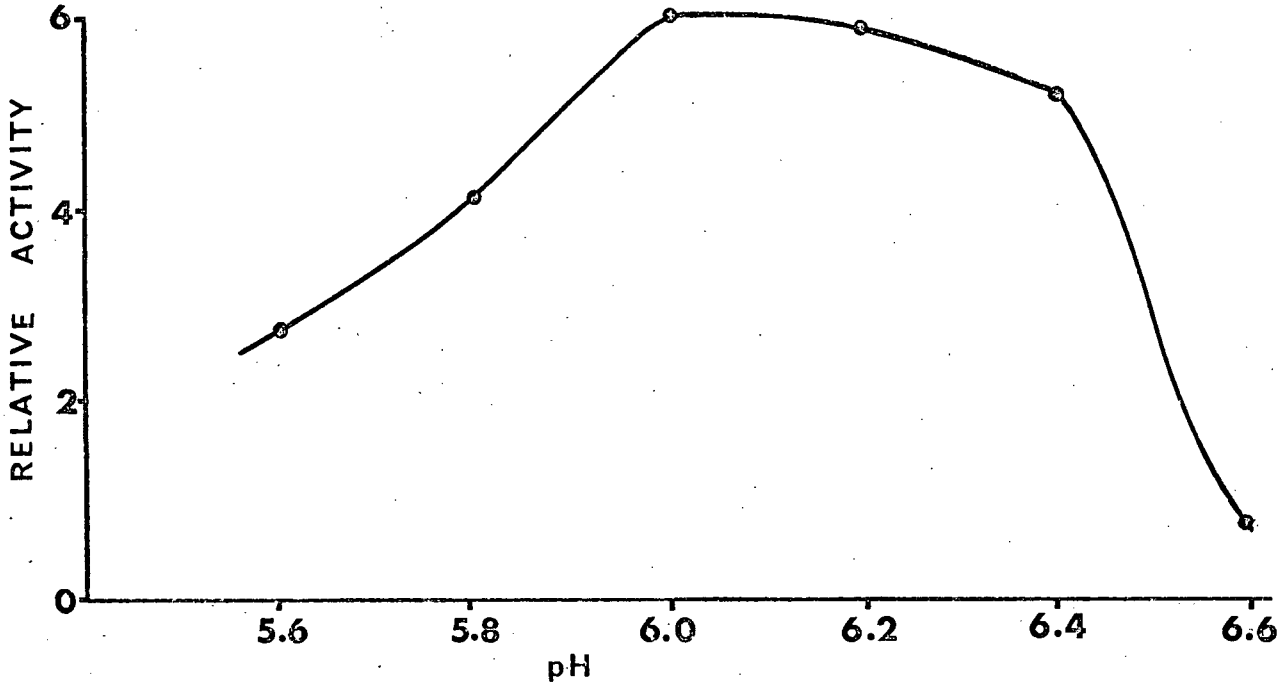


Fig. 4: Effect of pH of incubation mixture on the activity of tyrosine hydroxylase in brain

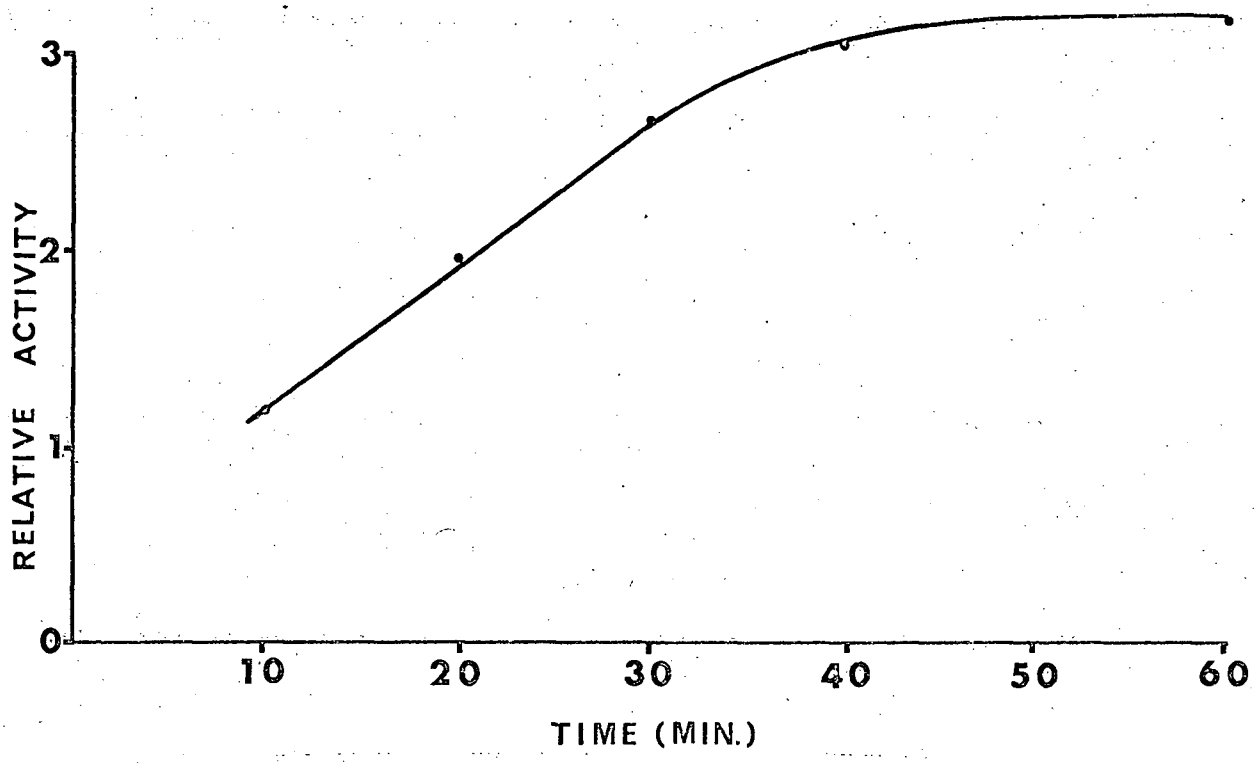


Fig. 5: Activity of Tyrosine hydroxylase with respect to time of incubation

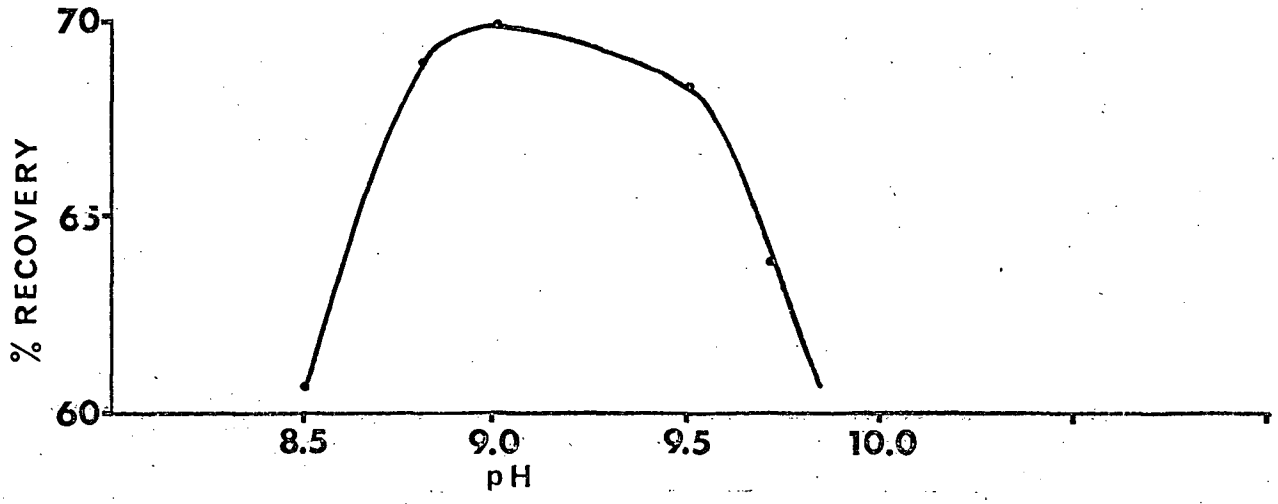


Fig. 6: Recovery of catechols from alumina at various pH's of the sample as it is placed on the column

TABLE 2

EFFECT OF $DMPH_4$ ON TYROSINE HYDROXYLASE ACTIVITY IN CAT, RABBIT AND RAT BRAIN

	Activity with $DMPH_4$ as Percent of Activity Without
11 Areas of Rabbit Brain	109% $\pm$ 17
18 Areas of Cat Brain	92% $\pm$ 21
36 Rat Brains	104% $\pm$ 9
Cat Adrenal Medulla	1030%

TABLE 3

EFFECT OF PHOSPHATE ADDITION ON SIZE
OF BLANK AND DOPA RECOVERY FROM ALUMINA COLUMN

	cpm			
Ml of Phosphate	0	1	3	5
Blank (150,000 cpm of Tyrosine-^{14}C)	1343	606	257	249
Dopa (6200 cpm)	4020	4136	4000	4210

specific for the catechol moiety at alkaline pH's. EDTA is added to prevent oxidation by heavy metals. Catechols are particularly susceptible to destruction at alkaline pH. The pH for maximum recovery is 9.0 as shown in Fig. 6. PO_4 is added because it was found to prevent the uptake of impurities in the original tyrosine solution which can give in a very large blank as shown in Table 3. The phosphate does not interfere with the Dopa recovery which is 65 - 70%. The samples consistently counted at 75% efficiency in the liquid-scintillation spectrophotometer.

b) Tyrosine Analysis

For the calculation of V_{max} for tyrosine hydroxylase it is necessary to know the endogenous tyrosine levels. Incubations are run below saturation and endogenous levels are high enough to affect the value.

i) Procedure

Tyrosine was determined by a modification of the procedure of Waalkes and Udenfriend (99). 0.2 ml of the sucrose homogenate were added to 0.2 ml of 30% TCA and 0.6 ml of H_2O (if sufficient tissue was available, the quantities were doubled to give more supernatant to work with). After centrifugation the supernatant was poured off and usually frozen before analysis. The analysate mixture consisted of 0.6 ml supernatant, 0.6 ml of 0.1% nitroso-2-naphthol in 95% ethanol and 0.6 ml of a mixture of 24.5 ml of 1:5 nitric acid and 0.5 ml of 2.5% $NaNO_2$. After thorough mixing the mixture was placed in a water bath at 55°C for 30 minutes. The unreacted 1-nitroso-2-naphthol was extracted with 2.5 ml of ethylene dichloride. The aqueous layer was placed in a small clear test tube and the fluorescence determined in a spectrophotofluorometer at 406 mμ activation and 570 mμs fluorescence. A sucrose-TCA blank and 1γ/ml standard solution were analyzed at the same time.

ii) Rational for Analysis:

Tyrosine has been shown to conjugate with 1-nitroso-2-naphthol under the conditions indicated (99) to form a fluorescent compound. Fluorescence is proportional to concentration over the range normally used (Fig. 7). Samples usually gave readings of

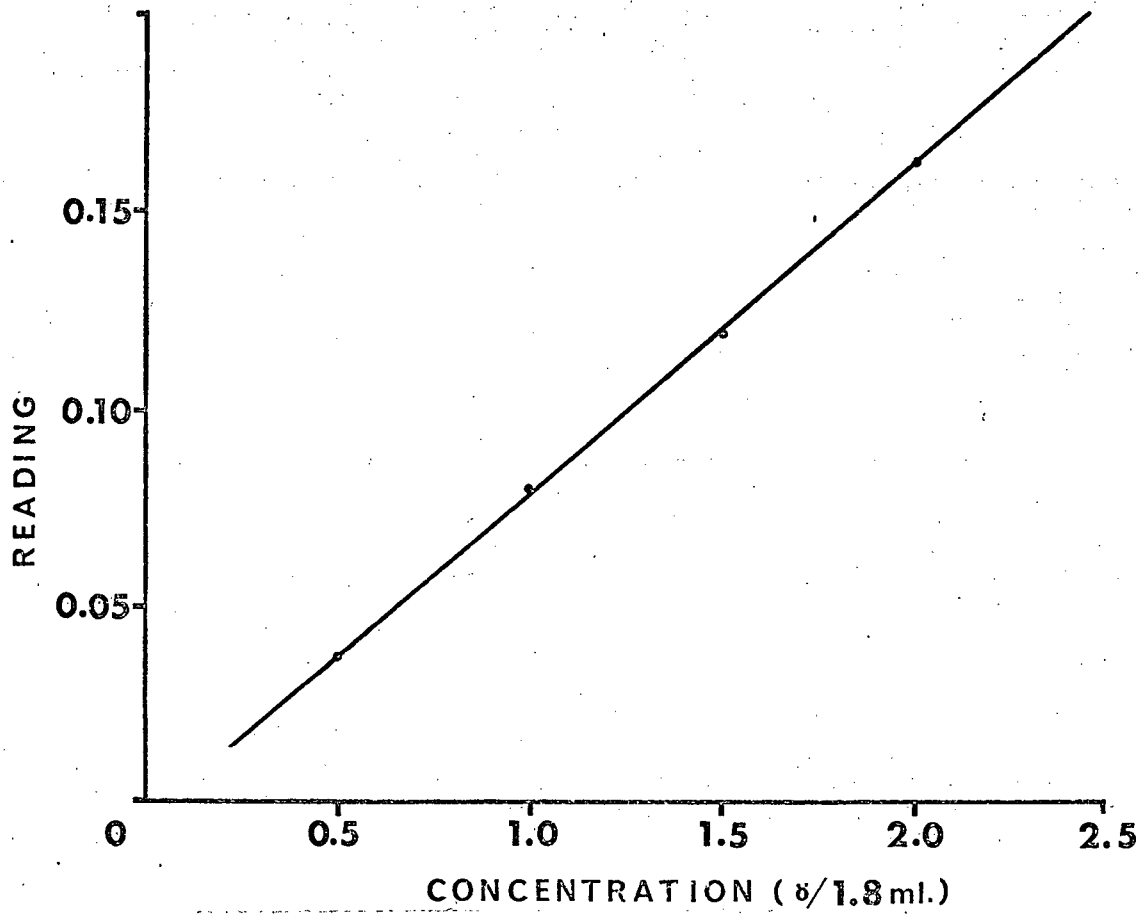


Fig. 7: Standard curve for tyrosine determination, fluorescence versus concentration

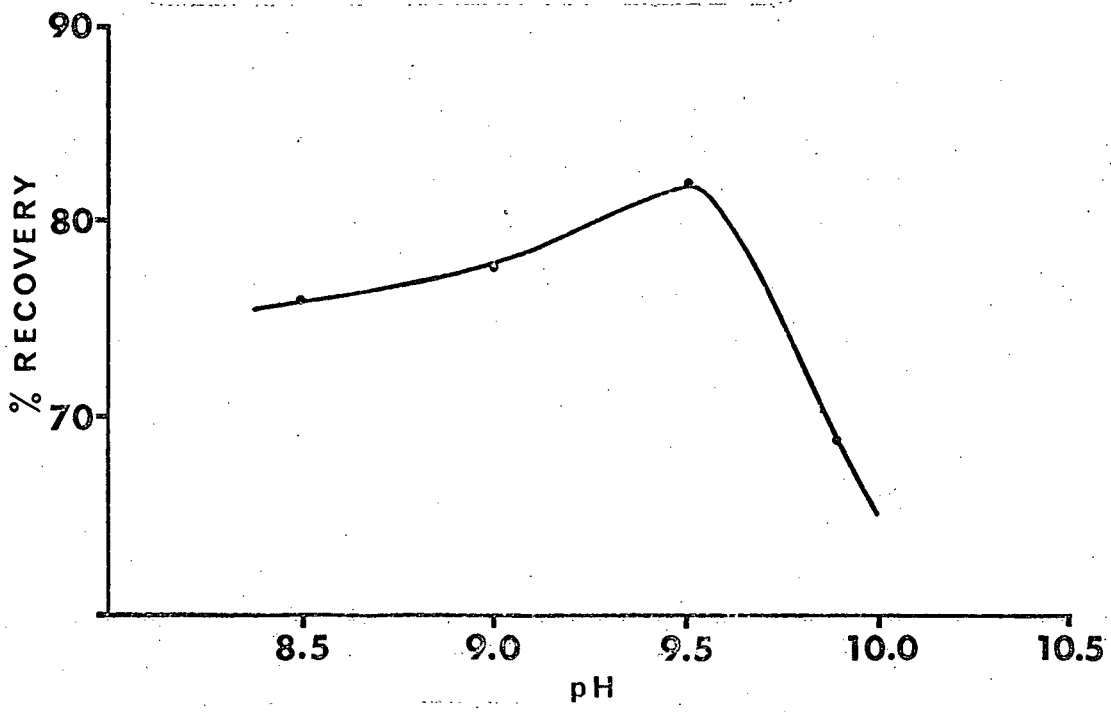


Fig. 10: Recovery of NA and DA from alumina with respect to pH of sample as it is placed in the column

0.06 - 0.15 fluorescent units and the blank was approximately 0.02.

c) Calculation of V_{max}

To calculate V_{max} for tyrosine hydroxylase it was necessary to determined the K_m value. K_m determinations were carried out using whole rat brain, cat and rabbit and cat and rabbit caudate. Incubations were carried out as previously described except that increasing amounts of cold tyrosine were added in the phosphate buffer. Plots were done according to the standard I/V vs I/S method and by the S/V vs S method as recommended by Dowd and Riggs (108). Fig. 8 is a representative plot for tyrosine hydroxylase in rat brain. Many determinations in this laboratory showed the average K_m for rat brain to be 0.45×10^{-5} (27). For cat and rabbit brain the K_m for tyrosine hydroxylase is of the order of 1×10^{-5}. Sample plots are shown in Fig. 9. These were the values used in calculation of V_{max}.

The V_{max} in mu moles of Dopa formed/gm of tissue/hr using the Michaelis-Menten formula:

$$V_{max} = v\left(1 + \frac{K_m}{c}\right)$$

K_m - in moles/l was determined experimentally for each animal as discussed above.

c - total concentration of tyrosine in moles/l of incubate. This includes radioactive and endogenous tyrosine.

v - mμmoles of Dopa formed/gm/hr. This is determined from the proportion of radioactive tyrosine converted to Dopa with corrections for the non-labelled tyrosine present.

Since the incubation volume, amount and activity of the radioactive tyrosine used, column recovery, counting efficiency and time of incubation were all kept constant, a formula could be

derived relating V_{max}, only, to the amount of tissue used, the tyrosine concentration in that tissue, the K_m for the reaction and the measured cpm.

$$V_{max} = \left[\left(\frac{54.3K_c + 46}{S} \right) + T \right] \quad 1.3 \times 10^{-4} \times \text{cpm}$$

$$K = \frac{K_m}{10^{-6}}$$

S = mg of tissue/incubation

T = endogenous tyrosine concentration in μg/gm

d) Noradrenaline and Dopamine Determinations

i) Isolation

Tissue was homogenized in a minimum volume of a 1:1 mixture of 0.4N $HClO_4$ and 0.2N HAc. If the tissue had been previously homogenized in sucrose for enzyme incubations the volume was noted and 0.2 ml of concentrated $HClO_4$ was added. The samples were cooled for 10 minutes, centrifuged for 10 minutes, and the supernatant was poured into a test tube containing 0.5 ml of EDTA. The precipitate was taken up in 2 ml of $HClO_4$: HAc mixture and the procedure repeated. At this point the samples were usually frozen until isolation on alumina, as previously described, except the mixture was taken to pH 9.0 - 9.5 before absorption on to the column. The eluant of 0.5N HAc was collected in a graduated test tube. 0.5 ml of 1.0M Ac buffer pH 6.0 was added and the pH was adjusted to 6.0 with NaOH. Water was added to make the final volume 3.0 ml. Standards of cold and radioactive catecholamines were run at the same time to determine percent recovery.

ii) NA Analysis

For each sample 3 small tubes were used:

1. sample
2. internal standard
3. blank

To each tube 0.5 ml of sample was added. Tubes 1 and 3 were buffered with 0.5 ml of 0.5M NaAc pH 6.4, and tube 2 with

0.5 ml of buffer containing 0.1 µg of NA. To all tubes 0.5 ml of 0.01N iodine was added followed in 4 minutes by 0.25 ml of 0.05N sodium thiosulphate solution. Tubes 1 and 2 were treated with 0.5 ml of a mixture of 7 ml of 5N NaOH and 0.5 ml of 0.5% ascorbic acid, tube 3 with 0.35 ml NaOH. The tubes were left standing in light for 90 minutes. Before readings were taken, in the spectrophotofluorometer, 0.15 ml of 0.5% ascorbic acid solution was added to the blank. The activation peak was 395 mµ and the fluorescent peak 505 mµ.

iii) DA analysis

All reagents must be at room temperature. Two large test tubes were used for each sample.

1. sample
2. internal standard

Two eluant blanks were run at the same time for each group of sample. To each tube 0.5 ml of sample was added to tube 1. 0.5 ml of 1.0M Ac buffer pH 6.0 was added, and to tube 2 0.5 ml of buffer containing 0.1 µg DA. Tubes were then treated with 0.25 ml of 0.01N iodine followed in 10 minutes by 0.25 ml of a 4.5N NaOH solution containing 25 mg/ml of anhydrous $Na_2S_2O_3$. Three minutes later 0.5 ml of 5N HCl was added to each. The tubes were left to stand in light for 12 - 24 hours and then read in the spectrophotofluorometer at activation peak 330 mµ and fluorescent peak 380.

iv) Rational for Catecholamine Determination

DA and NA were taken up on alumina as previously described for Dopa. However as shown in Fig. 10, the recovery was found to be maximum between pH 9.0 - 9.5. The recovery was more variable (60 - 80%) and cold standards were therefore run with each group. For very precise work internal radioactive catecholamine standards were used to determine the per cent recovery for each sample.

The fluorimetric analysis is based on the oxidation of catecholamines and their re-arrangement in alkaline to form fluorescent compounds (100). The equation for the noradrenaline reaction is:

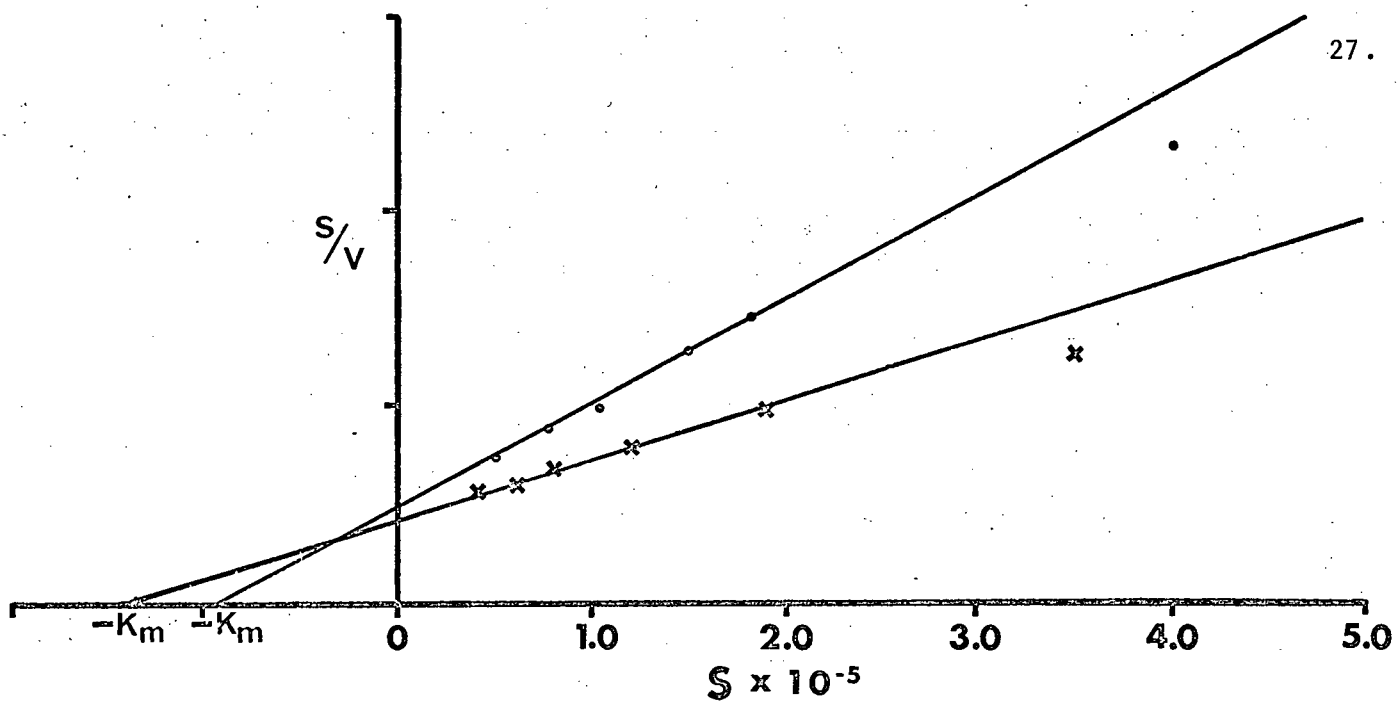


Fig. 9: K_m determination for rabbit (x) and cat (.) brain

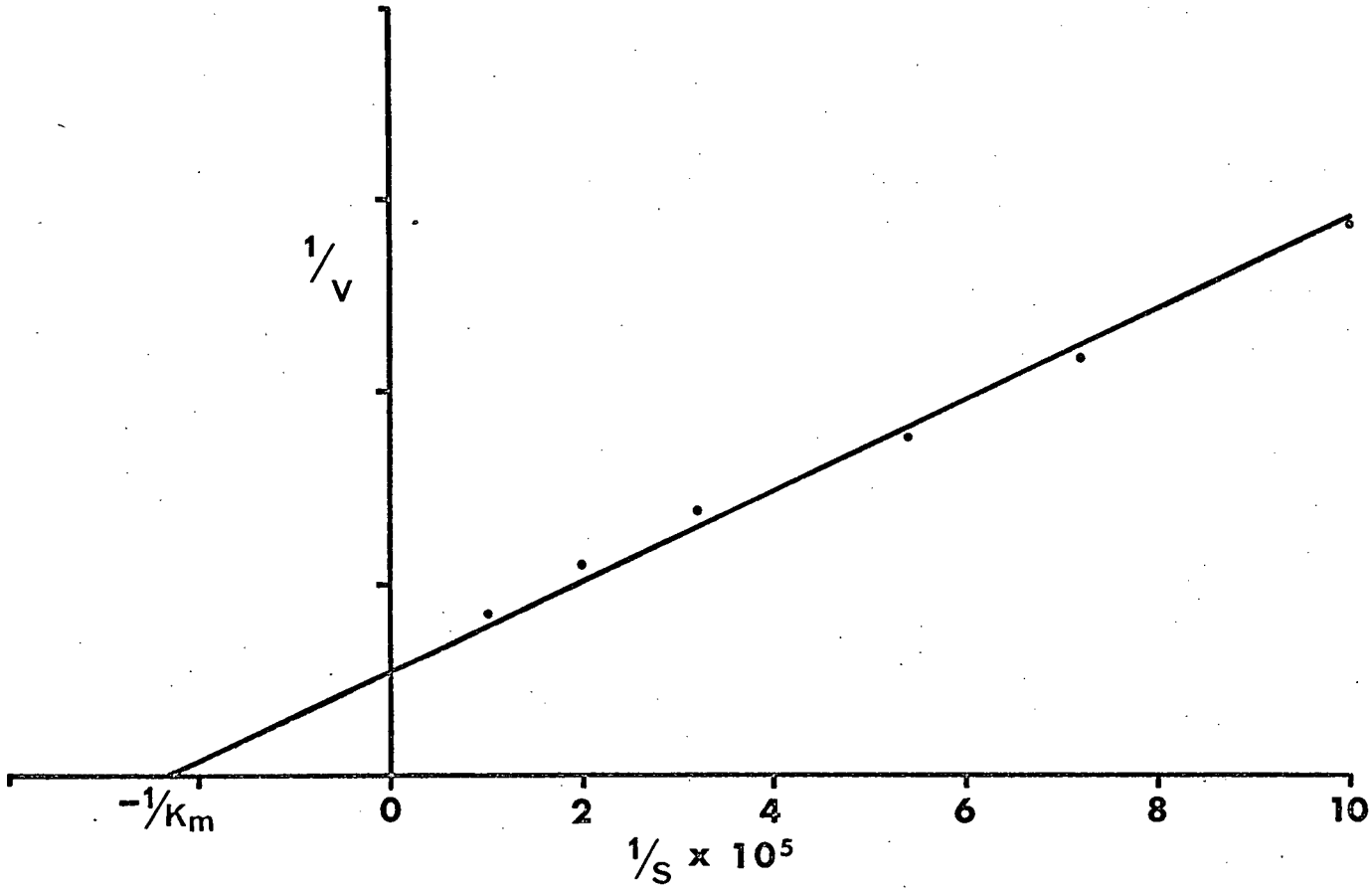
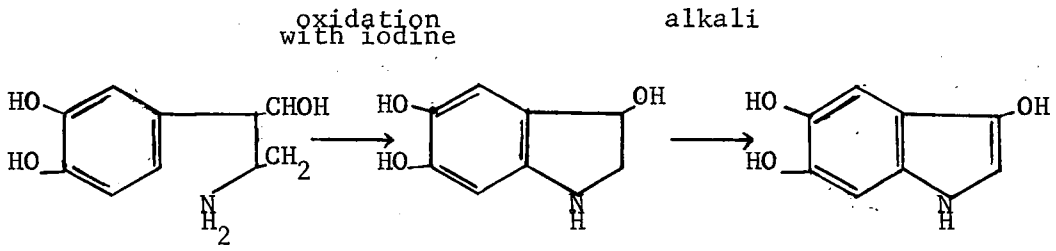


Fig. 8: K_m determination for rat brain



fluorescent

Because the fluorescent compound is unstable in alkali, ascorbic acid is added. Dopamine undergoes a similar reaction. The pH for DA analysis is lowered with HCl to decrease the wavelength of activation and fluorescence (to distinguish it from NA) and to intensify the fluorescence (6).

The optimum condition for these reactions had previously been worked out in this laboratory (101). From Fig. 11 it can be seen that there is a linear relationship between concentration of catecholamine and fluorescence. This method is very useful because very small quantities < 0.1 γ can easily be detected and measured accurately.

e) Separation of Noradrenaline, Dopamine and Some of Their Metabolites

Experiments were carried out to develop methods of separating noradrenaline, dopamine and their metabolites. Two methods were tried:

1. ion exchange chromatography as outlined in several references (102,103,104),
2. thin layer chromatography as suggested by Randeruth (105).

i) ion exchange chromatography - separation was carried out on Dowex 50 x 8, 200 - 400 mesh, which was kept under

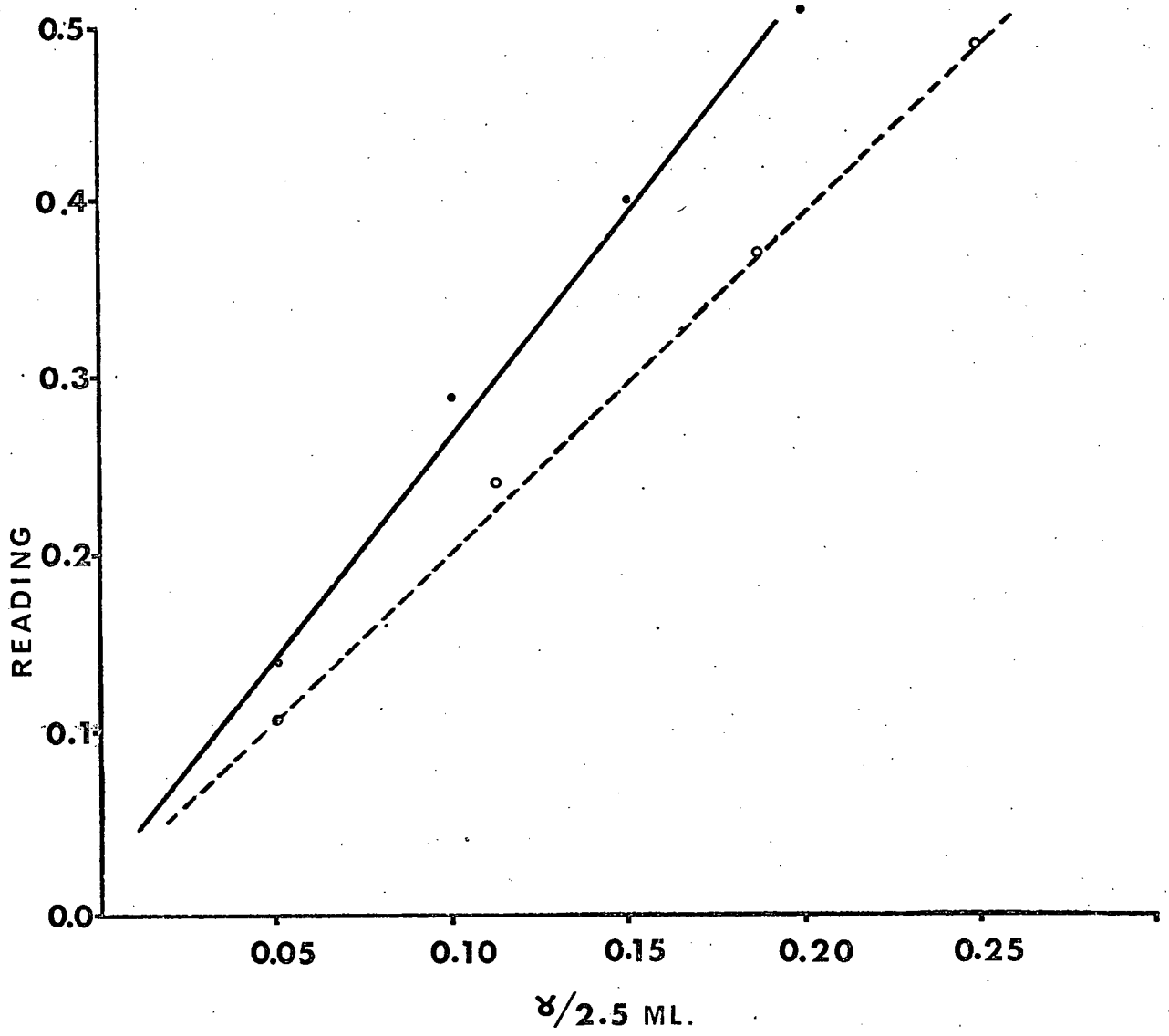


Fig. 11: Standard curve for NA and DA determinations. Fluorescence verus concentration

2N HCl prior to use. A small glass column, 4 mm in diameter was plugged with glass wool. The Dowex in 2N HCl was added so that the final height of resin was 5 cm. The resin was washed with distilled water until the washings were neutral (with pH paper) and then 10 - 15 mls of 1M NaAc pH 6.0 were passed through.

The amines (NA, DA and their O-methyl derivatives) were dissolved in approximately 2 ml 0.1N HCl for application to the column. Usually 25 γ of each were used plus various amounts of radioactive-^{14}C DA and NA. The resin was washed with 10 mls of water and 10 mls of 0.1N HCl.

Elution was carried out with 0.4N HCl at a rate of 0.2 - 0.3 mls/min. One ml fractions were collected and peaks of catecholamine fluorescence determined by measuring each fraction in the spectrophotofluorometer at activation peak of 285 mμ and emission of 330 mμ. A half ml aliquot of each sample was added to 10 mls of Bray's solution in a vial and counted in the liquid-scintillation spectrophotometer.

ii) Thin Layer Chromatography

Precoated sheets of polyamide were used as the stationary phase. The mobile phase was a mixture of isobutanol, acetic acid and cyclohexane (80:7:10). The tank was saturated with the solvent for 15 - 20 minutes prior to use. The chromatograms ran for 3 - 4 hours (8 - 10 cm above the origin). One - five μg of NA, DA, normetanephrine, methyoxytyramine, Dopa, tyrosine, 3,4-dihydroxy-phenylacetic acid and 3-methyl-4-hydroxymandelic acid were spotted. Those containing the catechol moiety (NA, DA, Dopa and dihydroxy-phenylacetic acid) were detected using a solution of ethylenediamine (1:1 with water) and examination under UV light. The remaining compounds were observed using a p-nitro-aniline reagent made by combining the following three solutions, in a 1:1:2 ratio just prior to use. These are:

1. 0.1 g p-nitro-aniline in 2 ml conc. HCl made up to

100 mls,

2. 0.2 g $NaNO_2$ in 100 ml H_2O, and,

3. 10% KCO_2 solution.

All compounds gave purple spots.

f) Catecholamine Activity in Rats in the Cold

i) Catecholamine determination in urine

Rats were placed in individual metabolic cages in the cold room (3^oC) and at room temperature. Both sets of rats were exposed to a cycle of 12 hours light and 12 hours darkness. Twenty four hour urine samples were collected and analyzed for NA and DA as previously described. Before isolation on the alumina the urines are taken to pH 4, heated in a boiling water bath for 10 minutes to hydrolyze catecholamines conjugates.

ii) Tyrosine hydroxylase activity in cold acclimatized rats

Experimental and control rats were housed as described in the previous section for periods of 4 hours to several weeks. The animals were sacrificed by a blow to the head, and the brains, adrenals, heart and spleen removed. The brain and adrenals were analyzed for tyrosine hydroxylase activity and for catecholamines. The hearts and spleens were analyzed only for catecholamines.

iii) Turnover studies for cold acclimitized rats

Rats housed as previously described were injected with the tyrosine hydroxylase inhibitors α-methyl-p-tyrosine, 100 mg/kg, and α-methyl-m-tyrosine, 100 mg/kg. The animals (2 experimental and 2 controls at each time period) were sacrificed at 2,4, and 6 hours after injection. The brains, adrenals, heart and spleen were analyzed for NA and DA. The log of the concentration of amine was plotted against time to determine turnover rates.

g) Effect of Altered Catecholamine Levels on Tyrosine Hydroxylase Activity in Rat Brain

Rats were injected with the MAO inhibitors pargyline and tranylcypramine, and the catecholamine depletors reserpine and guanethidine. The doses were pargyline 75 mg/kg followed by 5 mg/kg

12 hours later; tranylcypramine 30 mg/kg and 5 mg/kg; reserpine 25 mg/kg and 15 mg/kg and guanethidine 5 mg/kg both times. The animals were sacrificed 24 hours after administration of the first dose. The brains were analyzed for tyrosine hydroxylase and catecholamines.

h) Distribution of Tyrosine Hydroxylase in Brain

Rats, rabbits and cats were used in this study. Rats were sacrificed by a blow to the head, rabbits by cervical dislocation and cats by nitrogen asphyxiation. The brains were quickly removed and dissected into the areas as indicated in Table 9 and Fig. 12. The septal area in some of the cats was further divided as indicated in Fig. 13. The tissue was analyzed for tyrosine hydroxylase activity and the V_{max} for each area determined.

i) Study of Catecholamine Pathways in Cat Brain

Discrete electrolytic lesions were placed in the midbrain and diencephalon of cats using stereotaxic techniques. The stereotaxic atlases of Snider (106) and Jasper (107) were used in placement of the lesions as to anterior horizontal and vertical positioning. The size of the lesions could be varied by time and electric current intensity and by the number of needle placements. Seventy two hours after the operation the cats were sacrificed. The brain was removed and dissected into the areas described under distribution studies. Tyrosine hydroxylase analysis was done on all areas. Catecholamine determinations were done on caudate and septal area. Areas from each side of the brain were analyzed separately even in the case of bilateral lesions. The calculated V_{max}'s were compared with control values in the case of bilateral lesions and with the unlesioned side in the case of unilateral. The lesioned area was removed and placed in formaldehyde and given to the Pathology and/or Anatomy departments. There it was placed in a paraffin block, cut and stained by the Luxol Fast Blue method. The sections were examined by a neuroanatomist who reported on the position and extent of the lesion.

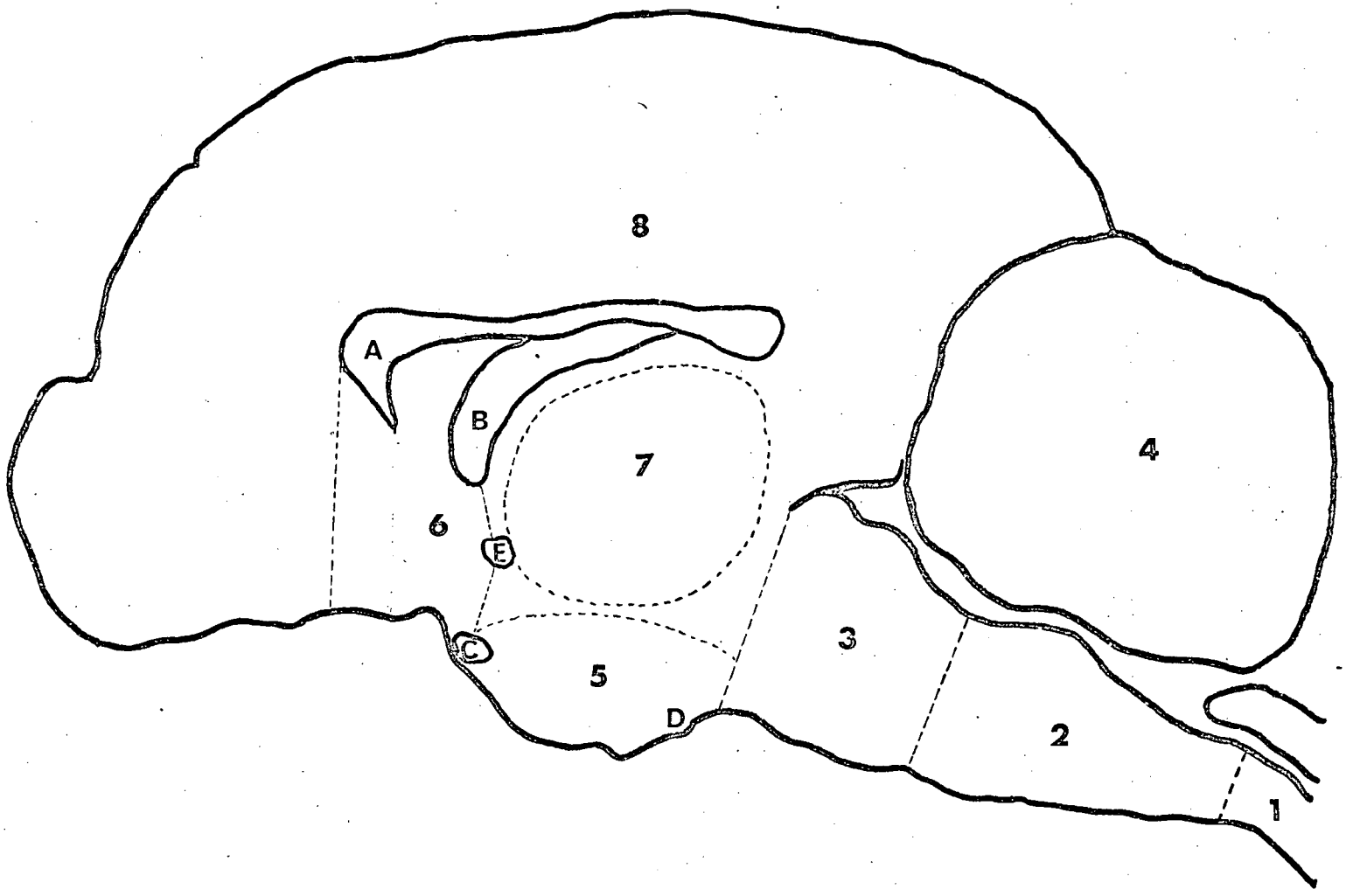


Fig. 12: Median sagittal section of brain to illustrate the method of dissection

Landmarks - A - corpus callosum, B - fornix, C - optic chiasm, D - mammillary bodies and E - anterior commissure

Areas - 1 - spinal cord, 2 - pons and medulla oblongata, 3 - midbrain, 4 - cerebellum, 5 - hypothalamus, 6 - septal area, 7 - thalamus, 8 - cortex

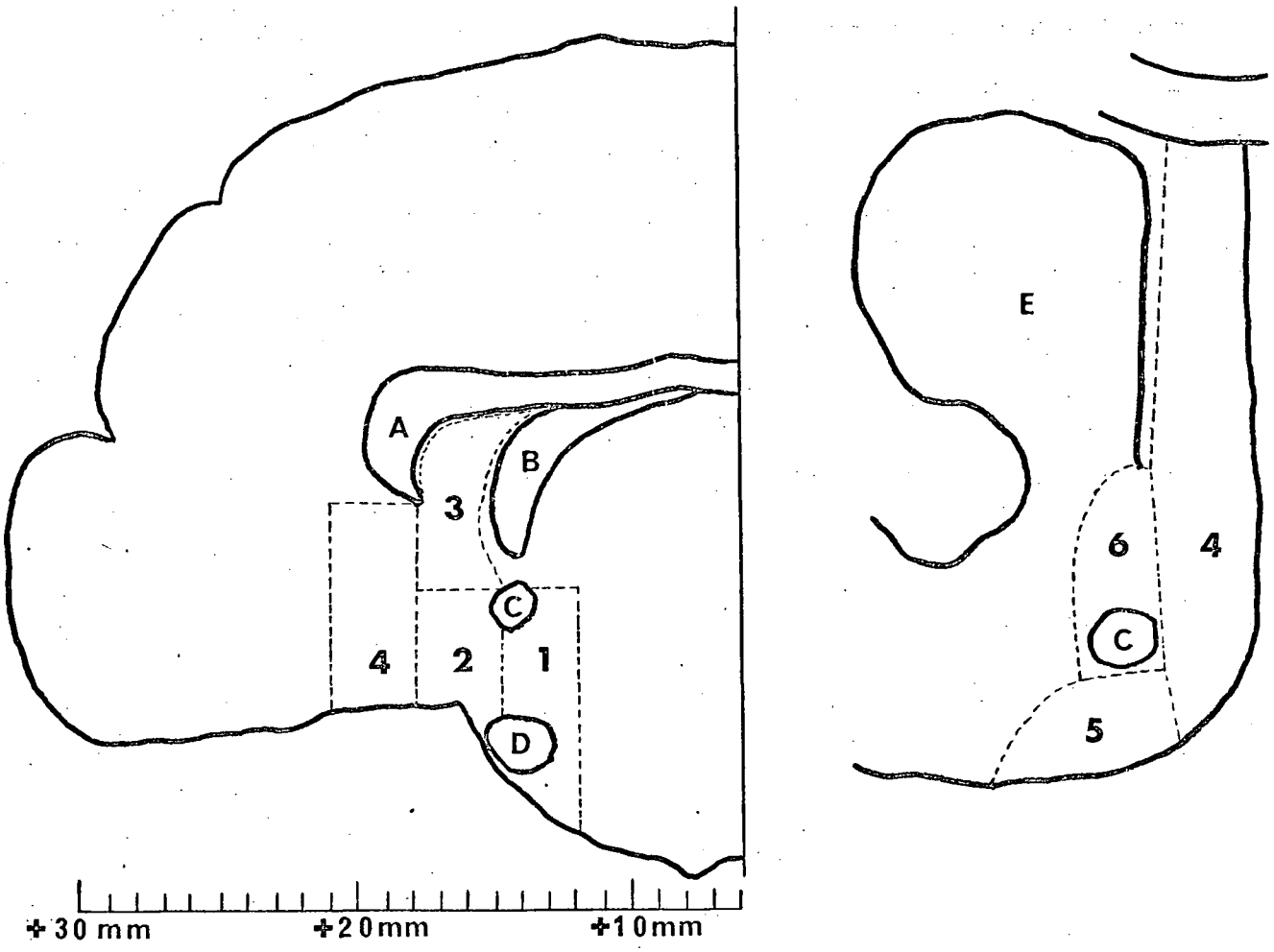


Fig. 13: Median sagittal and transverse section of cat brain to illustrate the subdivisions of the septal area

Landmarks - A - corpus callosum, B - fornix,
C - anterior commissure, D - optic chiasm

Areas - 1 - anterior hypothalamus
2 - preoptic area
3 - septal nuclei
4 - anterior septal area
5 - anterior perf. substance
6 - area of nucleus accombens

RESULTS

1. Separation of NA, DA and Some of Their Metabolites

a) Ion exchange chromatography

Since all the amines of interest (NA, DA, normetanephrine (NM) and methoxytyramine (MT)) have the same fluorescent peak, 285 mμ activation and 330 mμ fluorescence, the behavior of the compounds on the Dowex column was determined by running the amines one at a time and by using NA-^{14}C and DA-^{14}C. When elution from the column was carried out with 0.4N HCl, only NA was eluted in fractions 4 - 15, NM in 20 - 35 and DA in 25 - 45. If no NM was present, separation of NA and DA could be carried out more rapidly by using 2N HCl as an eluant after the NA had been removed from the column with 0.4N HCl. DA could then be collected, immediately after elution with 2N HCl began, in tubes 1 - 9 and MT could be eluted in tubes 11 - 20. These results are summarized by typical elution curves in Fig. 14 and 15. It was also found that tyrosine and Dopa could be separated from the amines by washing the column with 10 ml of phosphate buffer pH 5.0. The acid metabolites of DA and NA, homovanillic acid and 3-methoxy,4-hydroxymandelic acid, are probably not absorbed, or are only very weakly absorbed on this column. When no MAO inhibitor was added to an incubation mixture of brain homogenate containing DA-^{14}C, over 50% of the radioactivity appeared in the washings when this mixture was applied to the column. With an MAO inhibitor $<$ 1% was contained in the washings. Other investigators (102) have reported that these acid metabolites are not adsorbed in this type of column.

b)1 Thin Layer chromatography

R_f values obtained from thin layer chromatography of DA, NA and some of their metabolites are presented in Table 4.

2. Catecholamine Activity in Rats Exposed to Cold

a) Urine

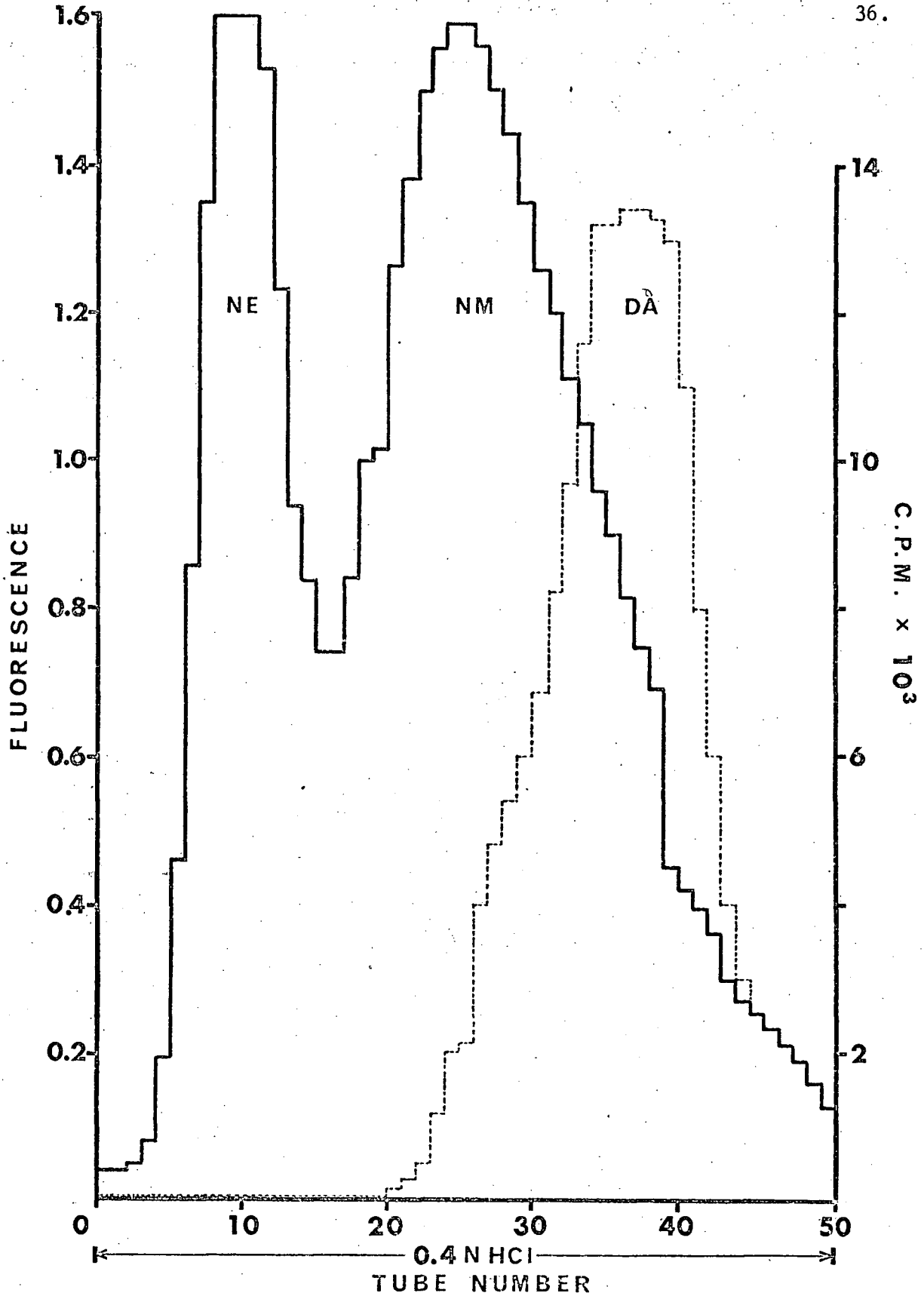


Fig. 14: Separation by ion-exchange chromatography of NA, NM and DA-^{14}C - fluorescence at 258 mμ/330 mμ — counts per minute for ^{14}C-DA.....

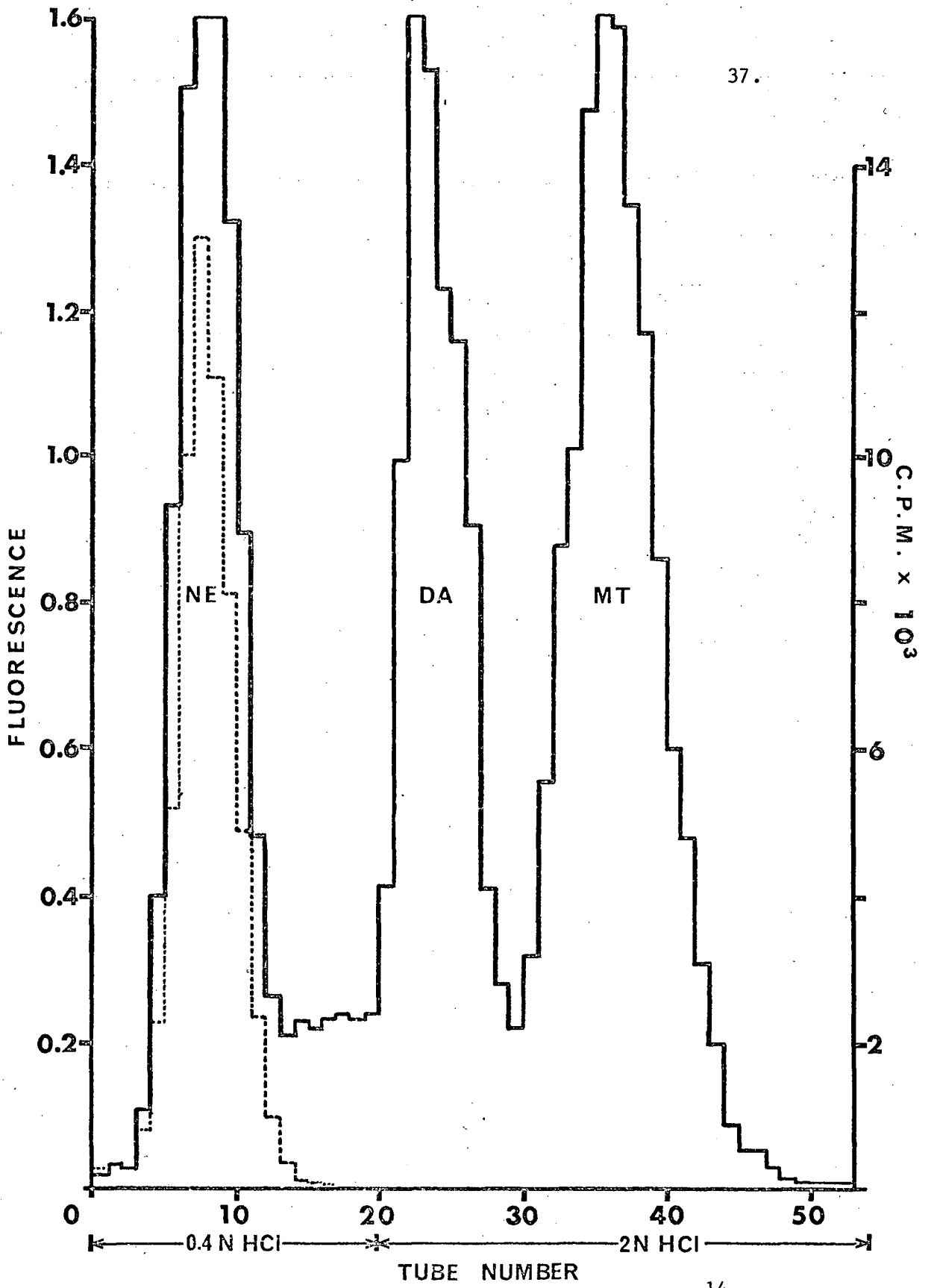


Fig. 15: Separation of NA, DA and MT and NA-^{14}C by ion-exchange chromatography fluorescence at 285 mμ/330 mμ —
Counts per minute for ^{14}-C-NA.....

TABLE 4

R_f VALUES FOR NA, DA AND SOME OF THEIR PRECURSORS AND METABOLITES ON POLYAMIDE

Compound	R_f (as obtained in this work)	R_f (as reported in 105)
Noradrenaline	0.29	0.42
Dopamine	0.39	0.51
Normetanephrine	0.69	0.69
Methoxytyramine	0.75	0.75
Dopa	0.16	-
Tyrosine	0.15	-
3,4-Dihydroxyphenylacetic acid	0.18	0.27
3-Methoxy-4-hydroxymandelic acid	0.19	0.35

As indicated in Table 5 there was a large increase (2 - 3 fold) in NA excretion by cold acclimatized rats. There was a slight increase in DA excretion. The increase occurred in the first 24 hours in the cold and the concentrations of DA and NA stayed at the same level for up to two weeks. The values given are the averages for urine samples from rats in the cold for from one day to two weeks.

b) Tissue

There was no significant difference in NA or DA contents of brain between normal rats and those exposed to cold. There was an increase (40%) of NA in adrenal but no change in DA. There may be an increase in spleen NA and a decrease in heart NA but the results on these tissues are not significant at the $p = 0.5$ level because of the large standard deviation. The results are presented in Table 6. Spleen and heart DA concentrations are not presented because they were very variable.

c) Tyrosine hydroxylase activity

There was no significant change in tyrosine hydroxylase activity per gram of tissue in either brain or adrenals of cold exposed animals as shown in Table 7. However, since the size of the adrenals increased in rats exposed to cold, there was an increase of 70% in tyrosine hydroxylase activity per adrenal which was significant ($p = 0.01$).

d) Turnover rates

In another attempt to assess the effect of cold exposure on catecholamine metabolism the rate of depletion of noradrenaline was measured after treatment with the tyrosine hydroxylase inhibitors, alpha-methyl-p-tyrosine and alpha-methyl-m-tyrosine. At the dosage used (100 mg/kg), dopamine levels in all tissue were not affected and so are not reported, and NA levels in heart with α-methyl-m-tyrosine. In brain, heart and spleen the curves for noradrenaline levels against time are very similar for cold acclimitized and for normal rats, indicating that the apparent noradrenaline turnover rates in these tissues are not markedly

TABLE 5

CATECHOLAMINE CONTENT OF 24 HOUR URINE SAMPLES OF RATS EXPOSED TO COLD*

	Control	Cold	% Significant Change at .01 Level
DA	100 ± 3% (11)†	112 ± 4% (10)	+12%
NA	100 ± 10% (14)	286 ± 2.5% (13)	+186%

* Expressed as % of control

† No. of animal in brackets

TABLE 6

CATECHOLAMINE LEVELS IN TISSUES
OF RATS EXPOSED TO COLD*

		Control	Cold	% Significant Change at .01 Level
Brain	NA	100 ± 13% (8)†	106 ± 18% (9)	-
	DA	100 ± 35% (5)	122 ± 8% (6)	-
Adrenal	NA	100 ± 4% (6)	140 ± 7% (6)	+40%
	DA	100 ± 36% (5)	93 ± 50% (15)	-
Spleen	NA	100 ± 27% (5)	185 ± 55% (6)	-
Heart	NA	100 ± 36% (5)	67 ± 31% (6)	-

* Expressed as % of control

† No. of animals in brackets

TABLE 7

TYROSINE HYDROXYLASE ACTIVITY
IN RATS EXPOSED TO COLD*

	Control	Cold
Brain	100 ± 15% (10) +	92 ± 17% (12)
Adrenals (per gm)	100 ± 20% (7)	140 ± 25% (7)
(per ad)'	100 ± 14%	170 ± 10%

* Expressed as % of control

\+ No. of animals in brackets

' adrenal

affected by the cold exposure. The curves for brain show a break at about 4 hours suggesting that the noradrenaline may have more than one turnover rate, or may be recovery as appears to be the case in heart, spleen and adrenals. (Fig. 16 (a) and (b))

Data for the adrenals are unsatisfactory in that the rate of depletion of noradrenaline in normal animals was more rapid after alpha-methyl-m-tyrosine than after alpha-methyl-p-tyrosine, possibly due to a plurality of actions. In the cold adapted animals the rates of depletion were approximately the same after both inhibitors, and indicated a faster turnover rate than in either group of controls. Further exploration of these phenomena was not done because of an insufficient supply of the inhibitors and the pressure of other programs.

3. Effects of Altering Catecholamine Levels on In Vitro Tyrosine Hydroxylase Activity

As indicated in Table 8, a number of drugs which significantly altered catecholamine levels in the brain had no effect on tyrosine hydroxylase activity of these brains as measured in vitro.

4. Distribution Studies of Tyrosine Hydroxylase in Rat, Rabbit and Cat Brain

In vitro activity of tyrosine hydroxylase in various regions of cat, rabbit and rat brain are presented in Table 9. Activity relative to the cerebellum is also shown. The caudate is by far the most active area being 5 x greater than the next two most active areas, the septum and anterior perforating substance. The caudate is almost 100 times more active in tyrosine hydroxylase than the cerebellum which is the least active area. The relative values as compared to the cerebellum, are quite consistent in the three species studied.

To show that there was a consistency in dissection, the average weight $\pm$ standard deviation is shown in Table 10.

Tyrosine values were also quite consistent for each area

TABLE 8

EFFECTS OF CERTAIN DRUGS ON
CATECHOLAMINE LEVELS AND TYROSINE HYDROXYLASE ACTIVITY
IN RAT BRAIN*

		NA	DA	Tyrosine Hydroxylase
Control	(4)†	100 ± 10%	100 ± 17%	100 ± 8%
Pargyline	(4)	180%	147%	99%
Tranyl-cypramine	(2)	155%	152%	94%
Reserpine	(4)	14%	40%	95%
Guanethidine	(2)	110%	89%	106%

* Expressed as % of control

† No. of animals in brackets

TABLE 9

DISTRIBUTION OF TYROSINE HYDROXYLASE

IN ADULT RAT, RABBIT AND CAT BRAIN *

Area	V_{max} †			Activity Relative to Cerebellum		
	rat	rabbit	cat	rat	rabbit	cat
Caudate	22.3 ± 5.5 (6)	70.9 ± 9.2 (5)	98.5 ± 9 (11)	56	101	70
Septal area	15.0 ± 2.4 (6)	20.3 ± 1.6 (4)	19.0 ± 5 (12)	1.1	29	13.5
Anterior Perforating Substance	-	-	28.6 ± 11 (4)	-	-	20
Amygdala	4.2 ± 0.5 (3)	-	4.2 ± 1 (11)	3.1	-	3.0
Hypothalamus	5.0 ± 1.6 (6)	3.7 ± 1.0 (5)	3.9 ± 0.7(11)	3.7	5.3	2.8
Thalamus		0.6 ± 0.1 (5)	2.7 ± 0.2(9)		0.9	1.9
Midbrain	3.6 ± 1.0 (6)	2.2 ± 0.5 (5)	3.1 ± 0.2(5)	2.7	3.1	2.2
Pons-Medulla	2.9 ± 0.3 (6)	1.7 ± 0.2 (5)	1.8 ± 0.5(13)	2.2	2.4	1.3
Cortex-Whole	3.1 ± 0.8 (6)	2.2 ± 1.0 (5)	2.5 ± 0.6(9)	2.3	3.1	1.8
-Visual	-	-	2.5 ± 1.5(9)	-	-	1.8
-Auditory	-	-	2.1 ± 1.8(9)	-	-	1.5
-Association	-	-	2.1 ± 1.6(9)	-	-	1.5
-Cingulate Gyrus	-	-	2.2 ± 1.9(9)	-	-	1.6
Hippocampus	1.8 ± 0.4 (6)	2.1 ± 0.6 (5)	1.6 ± 0.4(10)	1.3	3.0	1.1
Spinal Cord	1.2 ± 0.7 (5)	2.2 ± 0.9 (4)	-	1.0	3.1	-
Cerebellum	1.3 ± 0.4 (6)	0.7 ± 0.2 (4)	1.4 ± 0.2(4)	1.0	1.0	1.0

* No. of animals in parenthesis

† In mumoles DOPA/hr/gm of tissue

TABLE 10

TISSUE WEIGHTS OF DIFFERENT BRAIN AREAS*

Area	Rat	Rabbit	Cat
Caudate	33 ± 6	77 ± 7	416 ± 58
Septum	48 ± 23	70 ± 7	124 ± 26
Pons-Medulla	214 ± 30	801 ± 118	1408 ± 163
Midbrain	118 ± 15	536 ± 98	1074 ± 154
Hypothalamus	224 ± 67	115 ± 21	110 ± 30
Thalamus		400 ± 23	1278 ± 246
Hippocampus	96 ± 26	507 ± 74	582 ± 110
Amygdala	76 ± 14	-	400 ± 62
Cerebellum	256 ± 9	1144 ± 128	3438 ± 201
Anterior Perf. Substance	-	-	35 ± 9

* in mg.

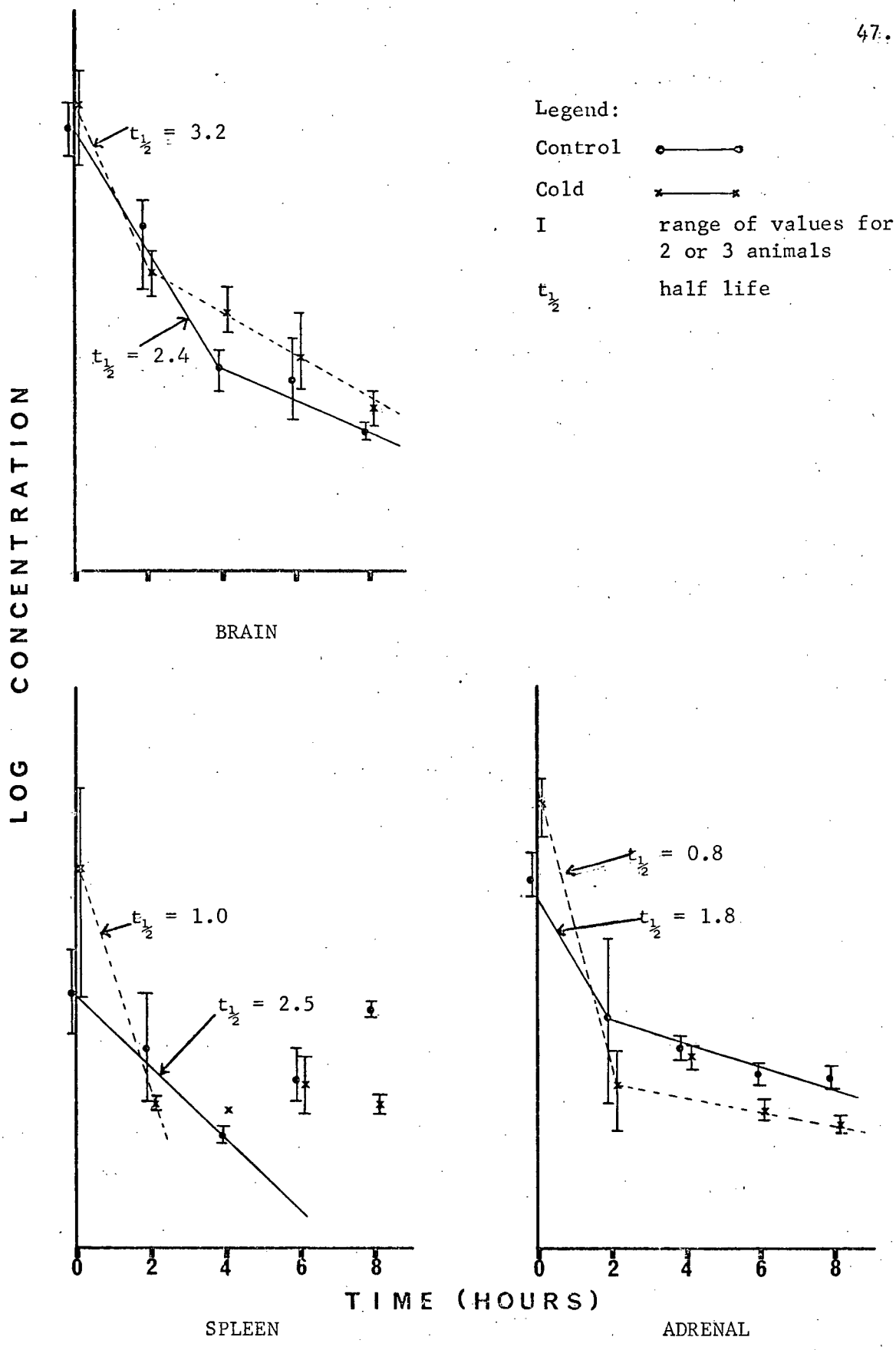


Fig. 16a: Depletion of NA with α-methyl-m-tyrosine organs of normal rats and rats exposed to cold

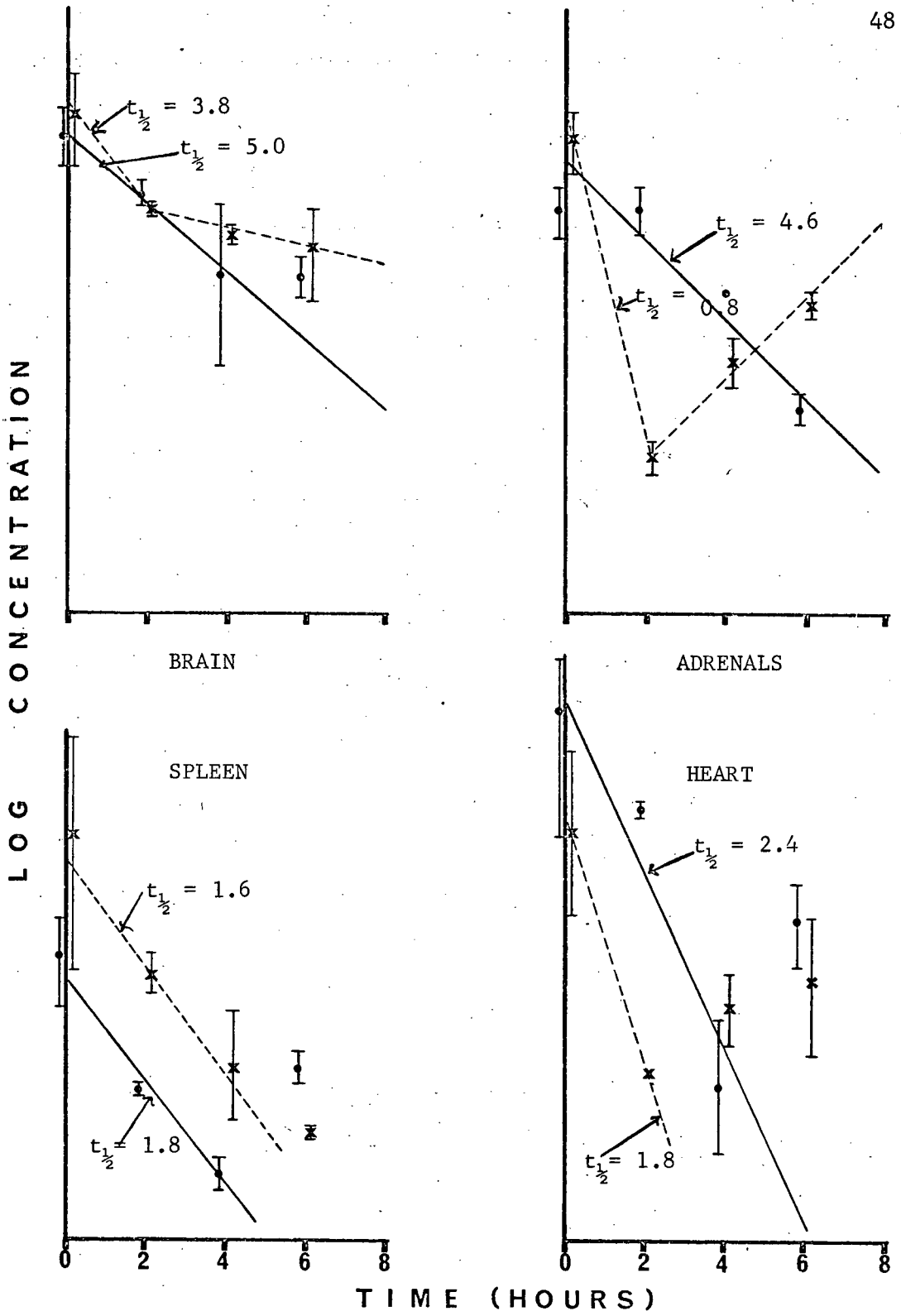


Fig. 16b: Depletion of NA with α-methyl-p-tyrosine from various organs of normal rats and rats exposed to the cold (for legend see Fig. 15)

as indicated in Table 11. There were slight differences between areas, but in general the values ranged from 15 - 30 γ/gm of wet tissue.

The tyrosine hydroxylase activities of the various subdivisions of the septal area for cat are presented in Table 12, along with the average weights and tyrosine values. The classical septal nuclei and preoptic area are relatively inactive. Most of the tyrosine hydroxylase activity of the septal area, as we defined it, is contained in the anterior portion just ventral to the septal nudei overlying the region of the nucleus accombens. This region may be part of the tuberculum olfactorum or nucleus of the diagonal band of Broca. The region of the nucleus accombens has about half the activity of the caudate and is therefore the second most active area we found in the brain. The anterior hypothalamus has about the same activity as does the whole hypothalamus.

5. Effect of Lesions on the Tyrosine Hydroxylase Levels in Various Regions of Cat Brain

Six main types of brains lesions were done:

1. floor of mid diencephalon,
2. posterior diencephalon (Fields of Forel),
3. habenular,
4. substantia nigra,
5. midline midbrain and
6. raphe

The locations of these lesions are shown in Fig. 17.

a) Lesions of diencephalon floor

Nine animals were lesioned at this level of the brain, 3 animals with medial lesions and 6 with lateral ones. The results are presented in Table 13. The medial lesions had little effect on tyrosine hydroxylase activity in the caudate; only one of the 3 cats showed a drop in activity to 63% of normal. However for these same lesions the septal tyrosine hydroxylase activity decreased

TABLE 11

TYROSINE CONCENTRATIONS IN VARIOUS AREAS

OF RAT, RABBIT AND CAT BRAIN*

Area	Rat	Rabbit	Cat
Pons-Medulla	17.3 ± 3.2	24.1 ± 10.1	13.4 ± 2.8
Caudate	28.8 ± 10.2	27.8 ± 5.2	24.6 ± 6.5
Septum	16.5 ± 6.3	24.9 ± 3.5	17.6 ± 6.0
Hippocampus	22.7 ± 7.1	28.3 ± 4.0	13.2 ± 3.2
Thalamus	20.8 ± 5.4	25.3 ± 6.2	14.5 ± 5.7
Hypothalamus		23.5 ± 5.1	19.8 ± 5.5
Amygdala	23.9 ± 5.1	-	16.1 ± 3.5
Cortex	15.7 ± 4.5	31.2 ± 4.8	14.8 ± 5.1
Midbrain	15.6 ± 4.2	25.8 ± 4.2	14.3 ± 7.0
Cerebellum	17.3 ± 5.5	22.9 ± 8.5	18.9 ± 4.6
Spinal Cord	22.7 ± 3.5	22.7 ± 3.7	-
Anterior Perforating Substance	-	-	23.3 ± 3.1

* ugm/gm ± standard deviation

TABLE 12

TYROSINE HYDROXYLASE ACTIVITY IN SUBDIVISIONS OF SEPTAL AREA OF CAT BRAIN

Region	V_{max}	Tyrosine	Tissue Wt.
	mumole/DOPA/g/hr	α/g	mg
Anterior Hypothalamus	5.9 ± 5.2	26.5 ± 5.1	25 ± 6
Preoptic	8.0 ± 3.8	21.2 ± 5.7	18 ± 5
Anterior Septum	32.6 ± 13.9	17.4 ± 3.5	37 ± 10
Region - Nucleus Accombens	61.0 ± 18.5	25.5 ± 7.5	28 ± 2
Classical Septal Nuclei	3.8 ± 0.9	17.1 ± 1.7	39 ± 18

TABLE 13

EFFECTS OF LESIONS IN FLOOR OF DIENCEPHALON ON TYROSINE HYDROXYLASE AND CATECHOLAMINES IN ROSTRAL AREAS*

Area	Medial+ (3)			Lateral (6)		
	Tyrosine Hydroxy-lase	DA	NA	Tyrosine Hydroxy-lase	DA	NA
Caudate	110	--	--	63, 34,	118, 41,	87, 105,
	63	74	89	63, 59	-, -,	-, -,
	104	105	77	15, 75	65, 41	110, 114
Septum	64	-	-	60, 65,	- -	- -
	14.5	-	-	125, 33,	- -	- -
	56.5	-	-	21, 103	- -	- -

* activity expressed as % of non-lesioned

\+ no. of animals in brackets

' medial caudate

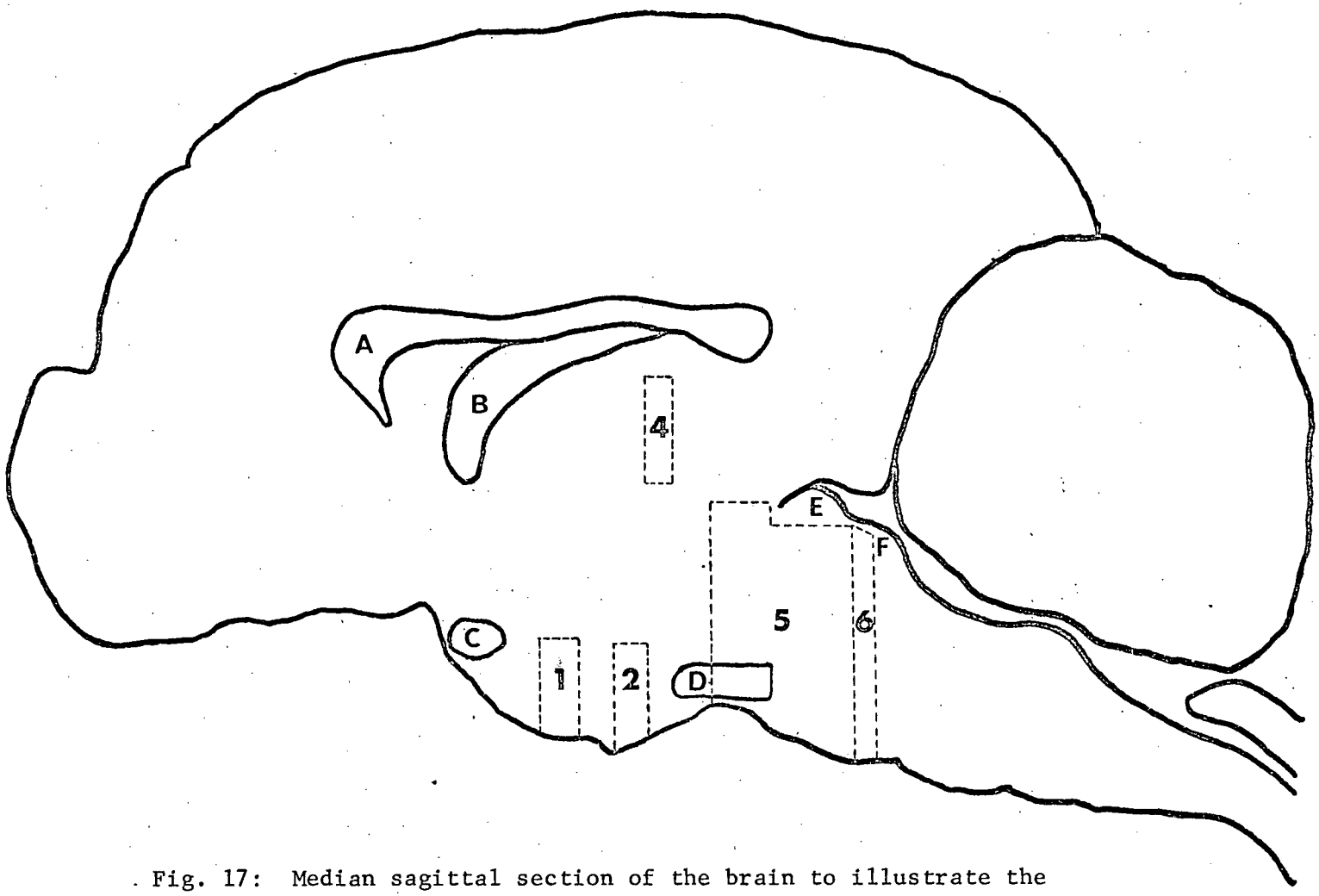


Fig. 17: Median sagittal section of the brain to illustrate the locations of the lesions of the diencephalon and midbrain.

Landmarks - A - corpus callosum, B - fornix, C - optic chiasm
D - interpeduncular nucleus, E - superior colliculi
F - inferior colliculi

Lesioned areas - 1. floor of mid-diencephalon, 2. posterior diencephalon
4. habenula 5. midline midbrain and raphe 6. cerveau isole

in all cats, to as little as 15% of normal in one case. Laterally placed lesions caused substantial decreases in activity of the enzyme in both caudate (15 - 65% of normal) and septal area (21 - 65%) in 4 of the animals. The other two lateral lesions showed decreases in caudate but none in the septal area.

There were no significant or consistent changes in tyrosine hydroxylase activity, with these diencephalic lesions in any of the other areas of brain, measured: i.e. amygdala, hippocampus, thalamus, hypothalamus, cortex and pons and medulla oblongata.

Caudate catecholamine levels were determined in 6 of these animals; septal area levels could not be measured because of insufficient tissue. There were no significant changes in caudate noradrenaline concentrations. In one animal where there was no significant change in caudate tyrosine hydroxylase activity, there was likewise no change in dopamine level. Four of the 5 cats which showed decreases in enzyme activity in the caudate also showed decreases in dopamine content, but these decreases were not as large as those of the enzyme (15% as compared to 45%).

No histological studies were done on the laterally placed lesions. One medial lesion, in which the septal tyrosine hydroxylase activity was 56.5% of normal, was confirmed histologically as a lesion of the midline floor of the diencephalon that did not extend far into the diencephalon. This lesion is shown in Fig. 18.

b) Posterior diencephalon - Fields of Forel

The 11 lesions done in this area can be divided into two groups:

i) large centrally placed one (4 cats) and

ii) small medially placed ones (7 cats).

With the larger lesions there were substantial decreases in tyrosine hydroxylase activity, to as low as 11% of normal, in both caudate and septal areas; the smaller lesions resulted in lower drops, to about 50% of normal. With both types of lesion the caudate and septal area decreased by approximately the same amount.

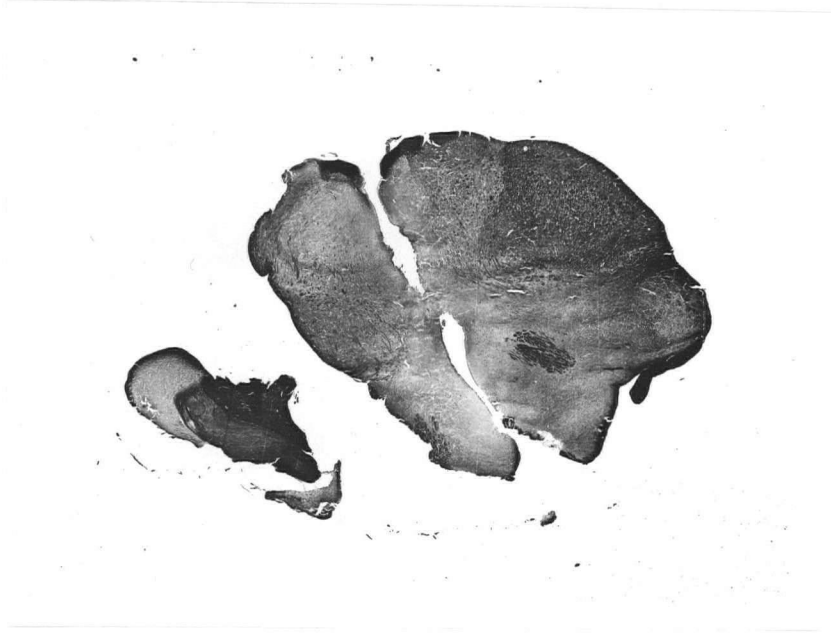


Fig. 18: Lesion in the floor of the mid-diencephalon

These results are presented in Table 14.

There were significant decreases in NA and DA concentration (to approximately 30% of normal) with the caudate of both of the cats in the large lesions on which amine analyses were done.

The only other areas of brain in which there were changes in tyrosine hydroxylase activity were the amygdala and hippocampus. Of the 11 cats lesioned, 5 had drops in activity of about 50% in the amygdala; and 9 animals had changes in activity in the hippocampus. The results for the hippocampus were however, very inconstant. Although the hippocampal activity was less than normal on the lesioned side in 6 cats, it was more than normal in a seventh. And in 2 other cases the hippocampus on the non-lesioned side showed no activity at all.

There were no histological studies for the large lesions. Three of the small medial lesions, which had caused decreases in tyrosine hydroxylase activity in both caudate and septal area, were confirmed histologically. Examples of 2 of these are shown in Fig. 19 and 20. Fig. 19 shows a lesion close to the midline in the ventral diencephalon at the level of the mammillary bodies. Fig. 20 shows a very discrete lesion at the same level but slightly more laterally placed. The entrance of the electrodes can also be seen in this picture.

c) Habenular

Eleven animals were lesioned in an attempt to destroy the habenular region, which would be at the same level as the Fields of Forel, but more dorsally located. Four of these habenular lesions were unilateral (â) and bilateral (b). On examination (histological) it was found that not all the lesions were in the habenular area. The biochemical results are presented in Table 15 with values for right and left sides of the brain with some bilateral lesions.

i) Three of the 4 cats with unilateral lesions had approximately 50% decreases in tyrosine hydroxylase in the septal

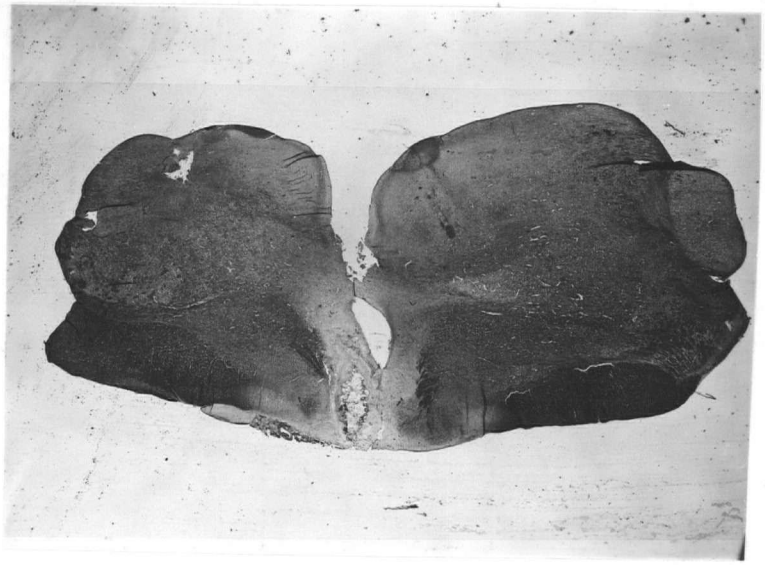


Fig. 19: Medially placed lesion in posterior diencephalon at the level of the mammillary bodies

Fig. 20: Lesion in the posterior diencephalon (only half of the brain)

TABLE 14

EFFECTS OF LESIONS IN FIELDS OF FOREL

ON TYROSINE HYDROXYLASE AND CATECHOLAMINES IN ROSTRAL AREAS*
(Only those animals showing changes presented)

Area	Large (4) (Central)			Small (7) (Medial)		
	Tyrosine Hydroxy-lase	DA	NA	Tyrosine Hydroxy-lase	DA	NA
Caudate	23	31	28	34, 36,	-	-
	45	27	33	59, 56,	-	-
	49			62	-	-
Septum	11	-	-	41, 57,	-	-
	17	-	-	43, 55,	-	-
	75	-	-	44, 65	-	-
Hippocampus	B1 (NL)	-	-	220, 67	-	-
	B1 (NL)	-	-	13, 58	-	-
	B1	-	-	B1, 50	-	-
Amygdala	49	-	-	60, 58,	-	-
	54	-	-	56	-	-

* Activity expressed as % of control

\+ no. of animal in brackets

B1 No activity (ie. equal to blank)

NL non-lesioned side

TABLE 15

EFFECTS OF ATTEMPTED HABENULAR LESIONS
ON TYROSINE HYDROXYLASE ACTIVITY
IN CAUDATE, SEPTUM, AMYGDALA AND HIPPOCAMPUS*

Area	Unilateral+(4)	Bilateral (7)
Caudate	109,108,114,85	11^{0}, 36,66,70
Septum	46,85,49,49	20^{0},27,71,60^{0},61
Hippocampus	64,75,100,39	17^{0},48,34,$B1^{0}$,35, 21^{0},B1,$B1^{0}$,9^{0},24
Amygdala	42,115,132,47	19,218^{0},150,185,236, 141

* expressed as % of control

\+ no. of animals in brackets

B1 no activity (ie. equal to blank)

0 designates from the same animal right and left side respectively

area, and amygdala but no changes in other areas. It was found however, that in all of these cats the habenular region had been spared with damage primarily dorsolateral to it. In 2 of the animals that had changes in activity in the septal area there were also evidences of damage on the midline of the diencephalon below the posterior commissure. An example of this type of lesion is seen in Fig. 21.

ii) Only 3 of the 7 bilateral habenular lesions resulted in decreases in tyrosine hydroxylase activity in the caudate and septal area. In one animal, showing a very large drop in activity to 10 - 30% of normal, the lesion was found on histological examination, to involve not only the habenular region but to extend laterally and ventrally from the ventricle almost to the floor of the diencephalon (see Fig. 22). Three of the other bilateral lesions that had no change in enzyme activity were confirmed histologically to be in the habenula area, only as shown in Fig. 23.

There were also changes in tyrosine hydroxylase activity in amygdala and hippocampus with the bilateral habenula lesions. In the same animal that had had large decreases in caudate and septal area the activity in the amygdala was 19% of normal; in 4 of the animals there was increased amygdala activity (141 - 236% of normal). The hippocampus activity was less than normal in 6 out of 7 animals.

d) Substantia nigra

Only 2 animals were lesioned in the substantia nigra since so much had previously been done in this area by other workers (51 - 56). As indicated in Table 16, the caudate in both cases showed the expected large drops, to as little as 2% of normal, in tyrosine hydroxylase activity in the caudate, as well as smaller decreases in DA (2% cf. 31%). The NA level was also below normal in one of the cats. There were also substantial decreases in septal tyrosine hydroxylase. Histologically, the lesions were

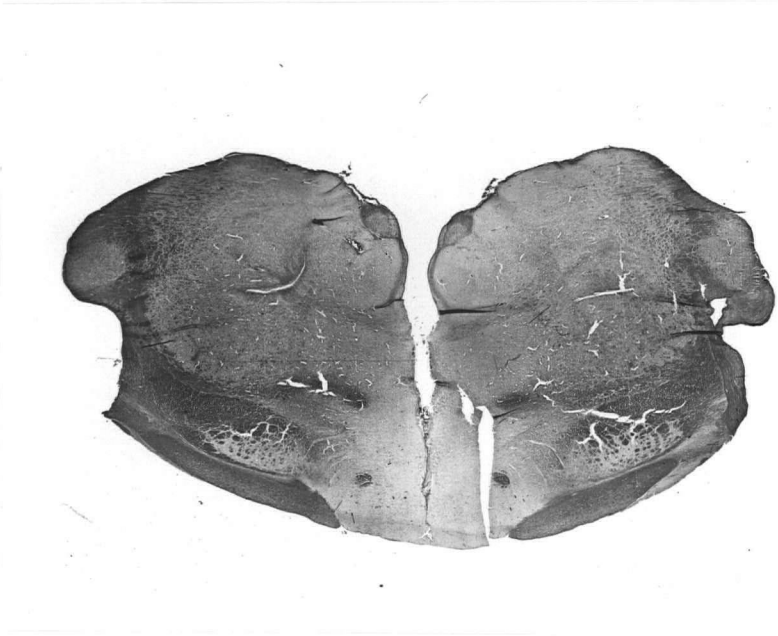


Fig. 21: Lesion made in an attempt to destroy the habenular region but with damage ventral to it.

62.

TABLE 16

EFFECTS OF SUBSTANTIA NIGRA LESIONS
ON TYROSINE HYDROXYLASE ACTIVITY AND CATECHOLAMINES
IN CAUDATE AND SEPTUM*

Area	Tyrosine Hydroxylase	DA	NA
Caudate	2, 5	31, 39	51, 50
Septum	20, 9.5	25, -	60,102

* expressed as % of control

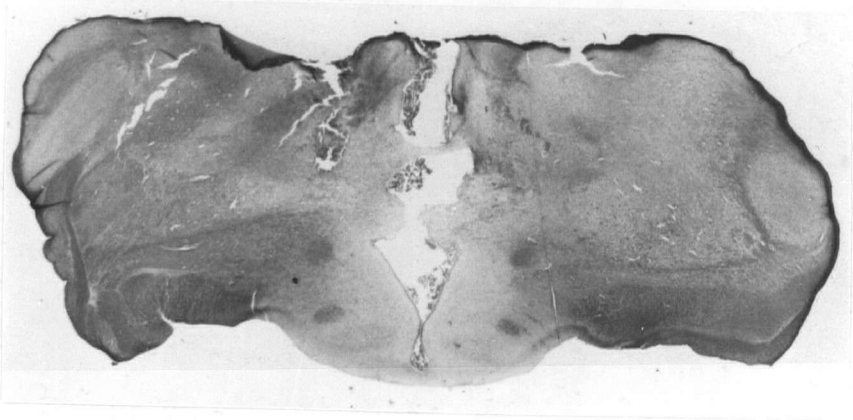


Fig. 22: Lesion in the habenular area that extended into the ventral diencephalon

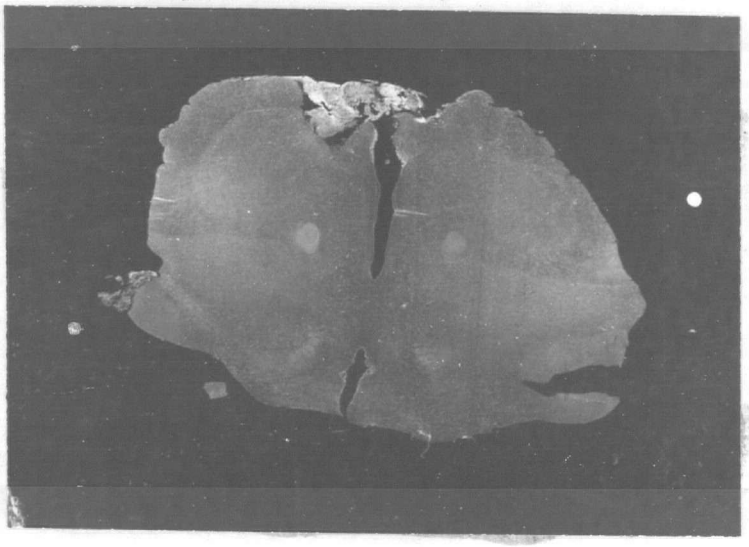


Fig. 23 : Lesion of the habenular only

confirmed to be in the substantia nigra, extending medial from it to the midline.

e) Midline midbrain

The 10 midline midbrain lesions were bilateral. There were two types of lesions:

i) cerveau-isole - lesions placed at a level between the inferior and superior colliculi and including most of the reticular formation on both sides of the midline (5 animals),

ii) lesions extending rostral to this cerveau isole under the superior colliculi and confined to the midline structures of the reticular formation (5 animals).

(a) The cerveau-isole lesions had little effect on tyrosine hydroxylase activity in either caudate or septal area, as shown in Table 17. Four of these lesions were confirmed histologically. A dorsally located lesion was described as destruction of the midline tegmentum of the midbrain, extending 2 mm on either side at the level of the caudal inferior colliculi, with perhaps a slight medial encroachment on the substantia nigra, but sparing the interpeduncular nucleus (Fig. 24). More ventrally located lesions of this type also destroyed the interpeduncular nucleus (Fig. 25).

(b) The lesions just rostral to this cerveau-isole produced very interesting changes in tyrosine hydroxylase activity in caudate and septal area. A lesion on the midline at the superior colliculi level, with little lateral extension, as shown in Fig. 26, produced no change in caudate or septal area activity (see Table 17). However a lesion in another animal, at the same level, but extending more laterally (see Fig. 27) produced drops in both caudate and septal area to about 50% of normal. The importance of lateral extent of the lesion was evident in one animal where histological examination showed that

TABLE 17

EFFECT OF BILATERAL MIDBRAIN LESIONS
ON TYROSINE HYDROXYLASE ACTIVITY IN CAUDATE AND SEPTUM*

Area	Cerveau Isole (5)+	Midline (5)	Raphe (4)
Caudate	60	56	115 0
	71	102	13 0
	95	110 0	106 0
	56	35 0	101 0
	70	30 0	125 0
		18 0	111 0
		13.5 0	59.6
		50	55.1
Septum	108	58	92
	103	96	Bl
	97	23 0	11.5
	70	20 0	76
	75	55 0	106 0
		28 0	62 0
		33	57.3 0
		74	68 0

* expressed as % of control

\+ no. of animals in brackets

Bl activity equal to blank

0 désignates from same animal - right and left side respectively

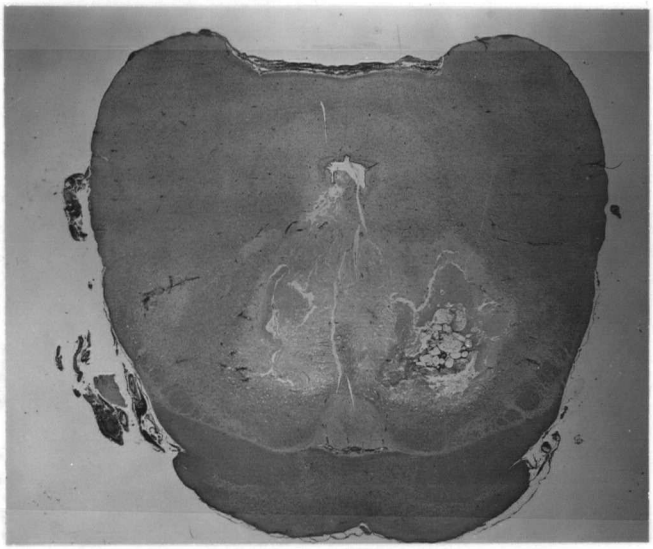


Fig. 24: Dorsal cerveau-isole lesion

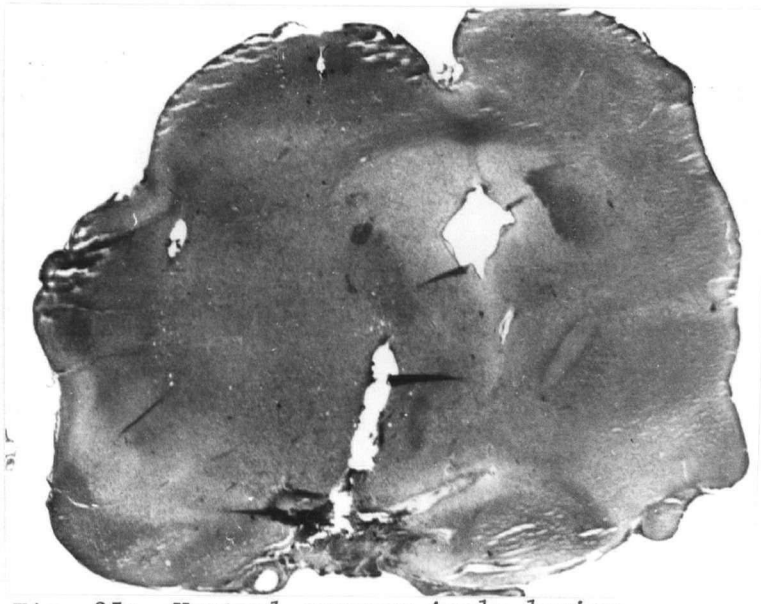


Fig. 25: Ventral cerveau-isole lesion

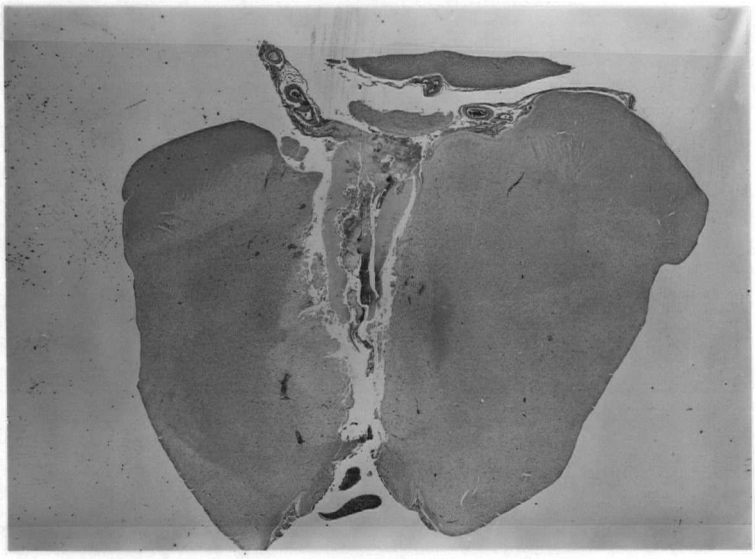


Fig. 26: Lesion of the midline of the midbrain with only slight lateral extension

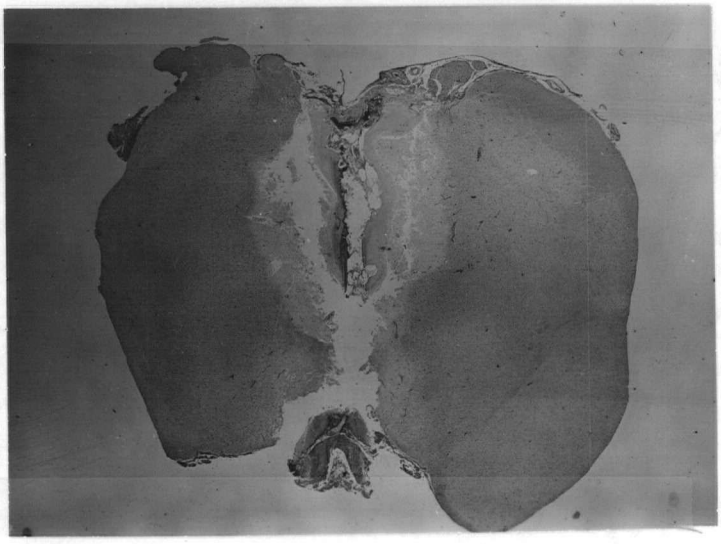


Fig. 27: Lesion of the midline midbrain with a large lateral extension

the midline midbrain tegmentum was destroyed more laterally in the right side than on the left (Fig. 28). In this animal the decrease in enzyme activity were on the left and right sides being 30% and 18% of normal respectively for caudate and 55% and 25% for septal.area The other two animals of this group had similar results, in that differences in the decreases in tyrosine hydroxylase activity on the right and left could be correlated with the lateral extent of the lesion as determined histologically. The caudate and septal area were not always comparable, in one animal the caudate tyrosine hydroxylase activity was normal on the right side while the septal activity was 20% of normal.

In all these midline midbrain lesions the damage extended from the aquaduct (and in some cases above it) to the floor of the midbrain including the interpeduncular nucleus, the substantia nigra and other peripheral nuclei were largely spared.

f) Raphe

The 4 raphe lesions were all confirmed histologically. These lesions did not extend as ventrally as those 5b but otherwise they were histologically similar, i.e. destruction of the midline structures of the midbrain, such as the dorsal raphe, medial longitudinal fasciculus and occulomotor complex, between the caudal inferior colliculi and caudal superior colliculi (see Fig. 29).

As shown in Table 17 tyrosine hydroxylase activity in the caudate and septal area could selectively be left unchanged or decreased to practically nil depending on the lateral extent of the lesion. As with the lesions of 5b type both caudate and septal activity could be affected or just septal area but never just caudate activity.

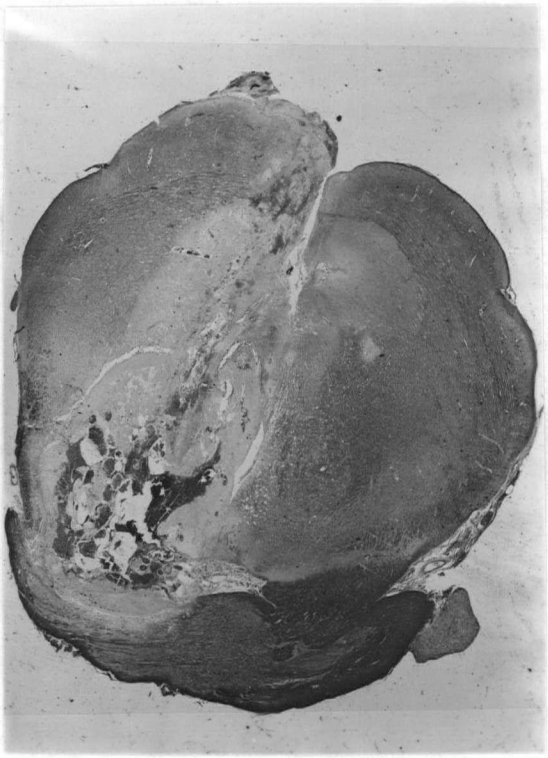


Fig. 28: Lesion of the midline midbrain with lateral extension of the lesion more on the right

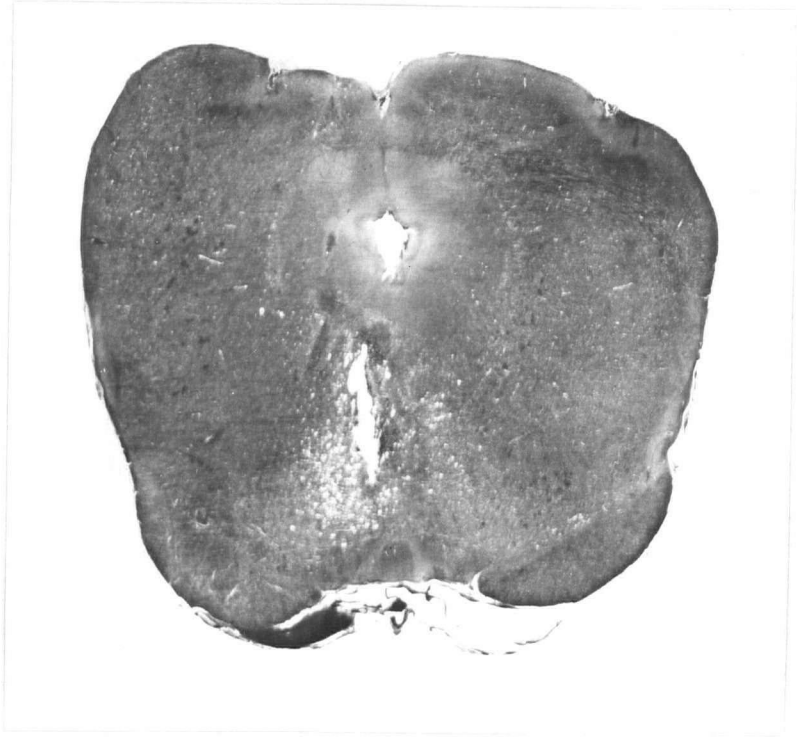


Fig. 29: Lesion of the midline midbrain in the dorsal raphe

DISCUSSION

1. Tyrosine Hydroxylase Activity In Vitro

The method, described in this investigation for determination of tyrosine hydroxylase activity *in vitro* has proven to be a rapid and precise one; rapid in that up to 80 incubations could be done in 2 days and precise in that duplicate incubations were within several per cent of each other. Accuracy also, is suggested by the fact that consistent results are obtained for each area of the brain (Table 9). Another method for measurement has been described in the literature (108,110) using tritiated tyrosine. The ^{3}H on the meta-position of the aromatic ring is released into the water during the reaction and can be readily measured. This method has been reported to give similar results to the ^{14}C-tyrosine method.

The values for tyrosine hydroxylase activity were in the range of 1 - 100 μmoles of Dopa formed/gm/hr for brain. This is of the same order of magnitude as reported by others (27,28) who obtained values in the range of 4 - 100 μmoles/gm/hr. The activity in adrenal is reported (28) to be 15 times that of brain; but in other peripheral tissue such as heart and spleen it is difficult to detect any activity *in vitro*. Sympathetic endings make up only a small proportion of such tissue which may account for the relative inactivity.

In vitro studies show differences in tyrosine hydroxylase in brain and adrenal. The conditions for maximum activity of crude adrenal homogenate (28) or of partially purified adrenal enzyme (23) are acetate buffer pH 6.0 in the presence of $DMPH_4$. The activity drops off after 20 minutes. We have found that brain tyrosine hydroxylase is most active in phosphate buffer pH 6.2, without $DMPH_4$ and with activity staying linear for at least 30 - 45 minutes. The enzymes may be different in brain and adrenal but the differences in *in vitro* activity could also be explained by differences in the ionic environment of the tissue

and the fact that there may be sufficient $DMPH_4$ in crude brain homogenate already. In support of this suggestion, Ikeda and Levitt (111) has reported that $DMPH_4$ enhances activity in purified dog caudate tyrosine hydroxylase. Also it was noted during these investigations of K_m values that at very high concentrations of tyrosine the curve 1/v vs 1/s was no longer linear without $DMPH_4$ but remained linear with cofactor. This may indicate $DMPH_4$ was becoming limiting at high tyrosine concentrations.

The K_m values obtained for tyrosine hydroxylase appear to be species and organ dependant. In brain the values as reported in this work ranged from 0.5×10^{-5} to 1×10^{-5} for the various species (cat, rabbit and rat) and it has been reported that for crude beef adrenal the K_m is in the order of 2×10^{-5} (28). Udenfriend has reported the K_m may range from 2×10^{-5} to 0.1×10^{-5} (23). He may have been referring to differences in species and organs.

Tyrosine hydroxylase has also been reported to be capable of converting phenylalanine to tyrosine in vitro in brain and adrenal tissue (112).

2. Separation of NA, DA and Some of Their Metabolites

a) Ion exchange chromatography

From the results (as shown in Fig. 15 and 14) this ion exchange chromatographic method is suitable for the separation of NA, DA and MT; NA, NM and MT, with incomplete separation of NM and DA. It is also a useful technique for the removal of the precursors of CA synthesis, tyrosine and Dopa, and of their acid metabolites from solutionsof DA and NA. Although different sizes of columns of Dowex and different elution schedules have been used by other workers (102-104) the types of separation achieved are similar.

This chromatographic technique has been used to separate the precursor DA from the product NA in the analysis of dopamine

β-oxidase (113,114), and for the study of in vivo metabolism of CA (30,31). Since tyrosine hydroxylase may not be the only centralling factor in CA synthesis it is useful to study other enzymes involved in CA synthesis and to investigate the in vivo metabolism. It would for example, be of interest to determine whether NA or DA is the major CA product in the septal area which is high in tyrosine hydroxylase.

b) Thin layer chromatography

In the original reference (105) this method of separation of CAs and their metabolites was claimed to be 100 times more sensitive than paper chromatography. With use of the detector ethylene diamine they were able to detect $< 0.05\gamma$ of CA.

In our hands the method was a good means of separating DA, NA, MT and NM for as indicated in Table 4 there was at least 0.1 R_f unit between each amine. The precursors tyrosine and Dopa and the acid metabolites of DA and NA had much lower R_f values and could be separated out from the amines that ran ahead of them. The R_f values obtained in this investigation are slightly lower than those reported (Table 4).

The separation of the amines obtained using thin layer chromatography is comparable with that of paper chromatography (115), and has the advantage of being more rapid, requires smaller quantities and no preliminary acetylation is required. It could be applied to studies of in vivo CA metabolism in place of the paper chromatographic techniques often used. Another thin layer chromatographic method using cellulose instead of polyamide has also given similar results (116).

3. Catecholamine Activity in Rats Exposed to Cold

The large increase in DA excretion in rats exposed to cold are in agreement with other reports (89,117). Leduc (89) claims a 5 fold increase in NA and a 40% increase in DA excretion in cold acclimatized animals.

However there are conflicting reports on the changes in

CA concentration in tissues of rats exposed to cold. Leduc (21) has reported no change in NA of adrenals and a decrease in concentration in heart and spleen; others have shown (117) a 40 - 50% increase in NA in adrenals and no change in brain NA content. The results presented in Table 6 (40% increase in NA in adrenals and no change in brain) would be in agreement with the latter report.

Leduc (89) has done the most extensive work with cold acclimatized rats. From his studies using adrenalectomized rats and ganglionic blocking agents, he concludes that NA released from sympathetic endings is the first, and adrenaline released from the adrenals is the second means of providing non-shivering thermogenesis in animals placed in the cold. Both NA and adrenaline have been shown to increase glycogen breakdown and cause fatty acid mobilization; the resulting increased metabolism could perhaps account for the required increase in heat production in cold acclimatized animals (118). Muscle and brown fat, which is richly innervated with sympathetic endings, have been proposed as sites of this increased heat formation (119).

From the results obtained in this investigation no definite conclusion could be drawn concerning tyrosine hydroxylase activity in rats exposed to cold. The increase in tyrosine hydroxylase activity per adrenal would indicate an increase in NA and adrenaline synthesis in that gland. The turnover studies with NA, however, could not substantiate this indication because consistent control values for NA turnover were not obtained. There are several possible reasons for the discrepencies between the values obtained with the two inhibitors. The doses may have been insufficient to produce complete inhibition of the enzyme (the dose of α-methyl-p-tyrosine used was insufficient to lower NA levels in heart). α-Methyl-m-tyrosine is also a Dopa decarboxylase inhibitor (120) while α-methy-p-tyrosine is not and this difference in action may have been a factor. The formation of

α-methyl NA from the inhibitors is also a possibility (121). This metabolite could interfere with storage sites of the CA as well as interfere with the analysis. In adrenal, heart and spleen there were also indications that the tissue was recovering from the inhibitors after 2 - 4 hours. Other investigators (122, 123) have reported much longer half-lives, in the order of 300 hours, for adrenals our work is not comparable to theirs. Glowinski (124) has shown that two thirds of the CAs in urine are from the periphery so it is probable that a large part of the excess CAs in urine of cold acclimatized rats is from peripheral tissue. Our results indicate that some may come from the adrenal. It does not appear to come from heart or spleen, but brown fat and other sympathetically innervated tissues, not considered in this investigation, may be an important source.

Even though no changes in tyrosine hydroxylase activity (Table 7) or in turnover rate of NA (Fig. 16 and 17) were obtained with brain tissue from rats exposed to cold, the brain cannot be excluded as important in cold adaption, probably in a regulatory capacity. It has been suggested (87) that NA in the hypothalamus and lower brain stem is important in control of body temperature. Since it is thought to have a hypothermic effect it might be expected that the hypothalamic NA might be decreased or its action be inhibited during cold adaption. The hypothermic action of NA is supposedly confined to very limited brain areas and measurements of CA concentration and tyrosine hydroxylase activity in whole brain may not reflect changes in these specific areas. From the distribution studies (Table 9) it can be seen that the hypothalamus and pons-medulla are only 1/20th and 1/100th as active as the caudate, and changes in activity in these areas would have little effect on activity of tyrosine hydroxylase in whole brain. Also in vitro assays of tyrosine hydroxylase may only measure maximum capacity of the tissue to produce CA, since all the factors affecting CA synthesis are not known (125). An altered in vivo activity of enzyme

might not be detected by in vitro measurement. Goldstein (126) has reported a slight increase in turnover of NA in brain of rats in the cold.

An interesting aspect of the turnover studies of NA in brain is the possibility that there are 2 turnover rates for this CA. The break in the curves (Fig. 5) may represent recovery from the inhibitor, but could also be attributed to a second turnover rates; Glowinski (22) has reported two turnover rates for NA of rat brain ($t_{\frac{1}{2}}$ = 3 hrs and 17 hrs). Since the $t_{\frac{1}{2}}$'s obtained are of the same order of magnitude (eg. in one case 3.8 and 18 hrs) it is possible that this phenomena is being observed. The two turnover rates would indicate two stores of NA; one store being labile ($t_{\frac{1}{2}}$ = 3 hrs) and probably in the cytoplasm as unbound NA; and the second store more tightly bound, possibly in the vesicles in noadrenergic nerve endings, and released more gradually during neuronal activity.

4. Tyrosine Hydroxylase Activity in Brain

a) Distribution

Like the CA tyrosine hydroxylase has a very definite distribution in brain (compare Table 1 and Table 9). DA is in high concentration in the caudate septal area and nucleus accombens; in moderate concentration in the hypothalamus and midbrain and in low concentration in the pons, medulla oblongata, thalamus, hippocampus, cerebellum and cortex. NA is high in concentration in the hypothalamus, moderate in concentration in the striatum and medulla oblongata and low in concentration in the cortex and cerebellum. Tyrosine hydroxylase has a high activity therefore and appears to be more closely related to distribution of DA rather than NA. The most active region of the septal area (anterior septal area) may correspond to parts of the tuberculum olfactorum which is high in DA and/or the nucleus of the diagonal band of Broca. The septal area, other than the classical septal nuclei, which has little tyrosine hydroxylase activity, is a poorly

defined region. Therefore it is difficult to relate the high tyrosine hydroxylase activity there to a specific structure.

The tyrosine hydroxylase distribution is in agreement with other investigation of CA synthesis in various regions of brain. The in vivo synthesis of NA in brain varies from 234 mμg/g/hr in hypothalamus to 33 mμg/g/hr in cortex, and rate of DA synthesis in caudate from preliminary reports (31) appears to be much higher. Injection of the radioactive precursor tyrosine, also shows different rates of synthesis; the striatum being more active with DA as the major product (10). Radioactive DA is converted to NA in the amygdala and hypothalamus but not in the striatum, indicating a different type of CA synthesis in various regions of brain as well as different degree (31). Work with brain slices and homogenates showed similar distribution of CA synthesis (127).

It had previously been demonstrated that tyrosine hydroxylase activity is contained in nerve endings (37). To substantiate this, it has now been shown that tyrosine hydroxylase is most active in areas containing a large number of nerve endings (eg. caudate and septum) as compared to the area containing cell bodies (eg. pons, medulla and midbrain). Also there is a loss of tyrosine hydroxylase activity when axons leading to these areas are cut (see lesion studies). It has been suggested (127) that tyrosine hydroxylase or a precursor is transported from the cell bodies to the nerve endings by axon flow.

The significance of this location is emphasized by the increases found in tyrosine hydroxylase activity in the areas of nerve endings (i.e. caudate, septal area, hippocampus and amygdala), during development of the brain (128).

The enzyme activity may be an indication of the formation of nerve endings or of useful dopaminergic and noradrenergic synapses. It is perhaps of interest that mentally retarded individuals do not respond to amphetamine, a drug that is thought to affect the CNS by its action on CA containing synapses.

b) As a control of CA synthesis

Although Udenfriend (28) has proposed that tyrosine hydroxylase in the rate limiting step in the synthesis of CA, it can not be assumed that it is the only controlling factor in their formation. Some potent in vivo and in vitro inhibitors of tyrosine hydroxylase do not cause a proportional decrease in CA content of tissue (43). Chlorpromazine is reported (129,130) to increase tyrosine hydroxylation in vivo, but has no effect on tyrosine hydroxylase activity in vitro (42). DA neurons recover more rapidly from α methyl-p-tyrosine inhibition than do NA terminals (7), and disulfuram (dopamine β-oxidase inhibitor) is more effective in reducing NA levels than α-methyl-p-tyrosine (131). Therefore there may be another control of NA synthesis at the dopamine B-oxidase stage.

In assessing the importance of tyrosine hydroxylase capacity in the control of CA synthesis, it is of interest to compare values for the in vitro enzyme activity of various areas of brain with the in vivo synthesis as obtained by Glowinski (9,30,31,32). As shown in Table 18, there is good agreement between the relative in vivo synthesis rates, and the in vitro tyrosine hydroxylase activities. Tyrosine hydroxylase is probably a better indication of CA activity in various regions of brain than CA concentrations which do not necessarily represent turnover, the cerebellum, for example, has a very low level of CA but has a fairly rapid turnover.

However, when absolute values of CA in vivo synthesis and tyrosine hydroxylase activity are considered the comparison is not so straight forward. Using Glowinski's figures that DA concentration in the striatum is 7.5 µg/gm, with a half-life of 1-4 hours, the in vivo synthesis would be 937 - 3750 mµg/gm/hr; for hypothalamus the formation of NA is 234 mµg/gm/hr. According to tyrosine hydroxylase activity figures about 15,000 mµg/gm/hr of DA could be produced in the striatum and about 750 mµg/gm/hr of NA in the hypothalamus. Therefore the in vitro activity of tyrosine hydroxylase is 3 to 16 times greater than that required for in vivo synthesis. Udenfriend's (27) suggestion that the hydroxylation

TABLE 18

RELATIVE TYROSINE HYDROXYLASE ACTIVITIES AND NORADRENALINE TURNOVER RATES IN RAT BRAIN

Area	Relative Tyrosine Hydroxylase	Relative NA Turnover *
Hypothalamus	3.7	5.6
Medulla	2.2	2.1
Cortex	2.3	0.9
Hippocampus	1.3	0.8
Cerebellum	1.0	1.0

* based on reference 31

is the rate limiting step is based on the much lower K_m for the hydroxylation as compared with the decarboxylase or with dopamine B-oxidase. But if there is an excess of enzyme over that required there must be some other factors or factor controlling its activity. One excellent possibility is feedback inhibition by the CAs. From work done in the periphery Stjarne (132,133) proposed that the NA content of sympathetic nerve stays relatively constant through varying degrees of activity because of the increased synthesis resulting from the removal of the inhibitor NA. This inhibition may be at the tyrosine hydroxylase or dopamine β-oxidase level. NA and DA inhibit brain tyrosine hydroxylase by about 40% and 70% respectively at 10^{-4}M (42). Normal CA concentrations in nerve endings have been estimated to be in the order of 8000 ug/gm (134) i.e. about 10^{-2}M but they are diluted out during the homogenizing process. Other possible limiting factors *in vivo* activity are:

1) a relative lack of cofactor or precursor that may become more readily available after homogenation;
2) non-ideal condition *in vivo* (eg. pH),
3) some endogenous inhibitor other than the CA.

It has been reported that slices of striatum show lower activity than homogenates; whereas some other areas had greater activity in slices (127). This may indicate that the state of the tissue is important in tyrosine hydroxylase activity.

An attempt was made in this investigation, to determine if altering CA levels, by drugs, changed tyrosine hydroxylase activity as measured *in vitro*. The results were negative but this may be due to the dilution factor mentioned above.

The fact that the hypothalamus is high in NA content but relatively low in tyrosine hydroxylase suggests that the enzyme in the hypothalamus is working closer to its maximum capacity than in the caudate. This may be related to feedback inhibition since DA is a better enzyme inhibitor *in vitro* than NA and there is a

higher concentration of DA in the caudate than in the hypothalamus.

Tyrosine hydroxylase is therefore a better indication of CA activity than CA levels but for a true indication of CA synthesis, other possible factors must also be considered.

Since the CA and tyrosine hydroxylase have a specific distribution in brain, it is assumed that they have a specific function. The CAs have been connected with emotion, behavior and learning but it is difficult to locate these anatomically. Perhaps, however, there is some relationship between the high concentration of DA, large capacity to synthesis CA and high turnover rate in the caudate and its possible role in motor function. On the other hand, the hypothalamus with its high NA concentration but lower capacity to form CA and smaller turnover rates, appears to be primarily concerned with autonomic function.

5. Tyrosine Hydroxylase Containing Fibers in Cat Brain

a) General consideration

As indicated in the results (Table 13 - 17) the caudate and septal area were the primary regions affected by the lesions, in midbrain structures of the cat. Decreases in activity in these areas could be determined with confidence since the variation around the mean for normal animals is relatively small (10 - 20%). Most of the discussion will be concerned with tracts to these areas. In many other areas it was difficult to determine changes since the counts for the controls were very low and there was a large variation around the norm. However where there were large and consistent changes such as found in the amygdala and hippocampus, which are probably meaningful.

Most other workers in this field leave the animals 2 - 10 weeks before sacrificing (49,59). It was found in this study that up to 80% decreases could be obtained in tyrosine hydroxylase activity within 72 hours of lesioning and the results were the same as in animals with similar lesions left for 6 days or 3 weeks. So for the majority of animals there was 72 hours between the time

of lesioning and sacrificing. The short interval however, made it difficult to observe chromolysis or extensive retrograde degeneration difficult, so no results as to possible location of cell bodies could be presented. Inconsistent results obtained with CA measurements may also be accounted for the short interval between lesioning and sacrificing. Moore and Heller (57) have shown the amines do not begin to decrease until the third day after lesioning and that there is a gradual reduction until the 12th day. They also claim that Wallerian degeneration is complete after three days. The indication then is that the nerve degenerates rapidly and that tyrosine hydroxylase is lost as rapidly as the nerve is destroyed, but that CAs are still present and undergo a more gradual destruction. Moore and Heller argue that the slowness of the CA decrease as compared to visible neuronal degeneration indicating a transynaptic effect. The Swedish workers (49) maintain, however, that the fibers are direct but are so small that the degeneration cannot be observed. The findings in this study would be in agreement with the latter theory.

b) Fibers in the diencephalon

i) Mid-diencephalon

From the results in Table 13, the following conclusions can be drawn:

1. CA containing fibers going to the caudate and septal area are present at the level of the mid third of the diencephalon.

2. fibers going to the caudate are lateral to those going to the septal area.

3. fibers to the caudate may be going primarily to the medial part.

4. at this level at least 85% of the fibers to the septal area can be account for.

Therefore in this lateral hypothalamic region of the mid-diencephalon there is some distinction between caudate fibers (lateral) and septal area (medial), but the separation is incomplete.

Moore and Heller (58) also obtained decreases in NA and dopa decarboxylase in the caudate and septal areas with lesions in

the same area (lateral hypothalamic, complete transection of the MFB), as well as decreases in the cingulate gyrus, hypothalamic region, hippocampus and amygdala. No consistent changes in tyrosine hydroxylase were obtained for these latter areas.

The results of this investigation in the diencephalon correlated with the findings of Swedish workers (49,50). With lesions of the MFB at the levels of the anterior commissure, they obtained decreases in DA in the tuberculum olfactorum and nucleus accombens, as well as NA changes in the hypothalamus, preoptic area, septal area and cingulate gyrus, but the striatum showed no change. They consider that the fibers to the caudate leave the MFB in the posterior diencephalon to enter the internal capsule and terminate primarily in the lateral and ventromedial caudate, but they do not account for fibers to the dorsomedial caudate. Our results would indicate that the fibers to the caudate go to at least the mid-diencephalon and that these fibers may be going primarily to the medial caudate. This would be in agreement with Poirier and Sourkes (53) who describe DA fibers to the striatum of cats and monkeys going more rostrally to those described by the Swedes. Using classical neuroanatomical techniques fibers in the MFB, lateral hypothalamic region have been shown to go to the caudate, diagonal band of Broca, hypothalamus, septal nuclei, hippocampus, amygdala and thalamus (64,65,66).

In attempts to correlate these types of lesions with behavior, it was found that lesions of the MFB caused increased escape latency time during learning (135,136).

ii) Posterior diencephalon

Lesions in the caudal portion of the diencephalon at the beginning of the MFB did not separate fibers going to the caudate from those to the septal area. Even with very precise lesions as shown in Fig. 20 medially or laterally placed lesions produced similar decreases in tyrosine hydroxylase in both areas (Table 12). AT this level in rats the Swedish group (62) were able to determine NA fibers to the diencephalon were most medially located, that DA to nucleus accombens and tuberculum olfactorum were slightly

lateral to the NA fibers and that these DA fibers to the caudate were most laterally located. We could not confirm these findings.

AT least 80% of the fibers to the caudate and septal area must pass through the Fields of Forel region; the majority of caudate fibers presumably have not yet left the MFB to enter the internal capsule as they are thought to do (49).

True habenular lesions appeared to have no effect on caudate and septal area tyrosine hydroxylase. Classical neuro-anatomical methods have shown some reciprocal connections between the habenular and septal nuclei (but these nuclei are relatively inactive in tyrosine hydroxylase) (Table 12). Those lesions which resulted in a change were not true habenular lesions but probably extended more ventrally into the Fields of Forel. It is of interest that in 3 of the 4 cats there were 50% decreases in septal area tyrosine hydroxylase with no change in the caudate. These lesions were more dorsal than those described previously, so fibers going to the septal area might be more dorsal than those to the caudate.

In both these posterior diencephalon lesions, (Fields of Forel and habenula) there appeared to be significant and consistent changes in amygdala and hippocampus tyrosine hydroxylase. Reciprocal connections between these areas and the reticular formation have been described (66,65). Some of these may be crossed, as a possible explanation for the decreases in activity obtained on the lesioned side. As previously mentioned Moore and Heller and the Swedish groups have described changes in CA concentrations in these areas with MFB lesions.

c) Fibers in the midbrain

The midbrain area would appear to be the critical region for the origin of CA containing fibers ending in the caudate and septal area.

i) To the Caudate

From these results it would appear that the substantia nigra is the origin of 80 - 90% of the fibers to the caudate. Lesions caudal to the substantia nigra eg. cerveau-isole, produced

no significant changes in tyrosine hydroxylase of the caudate, thus establishing the caudal extent of origin of fiber to the nucleus. Some midline lesions of the midbrain that did not affect the substantia nigra could decrease caudate tyrosine hydroxylase 20 - 30% of normal, the fibers apparently course medially from the substantia nigra as they also go rostrally.

Using their histochemical techniques on rats the Swedish groups (51) demontrated this nigro-striatal DA tract from the substantia nigra through the crus cerebri into the caudal MFB and then turning lateral through the internal capsule to the caudate and putamen. They found a direct relationship between the extent of substantia nigra destruction and loss of DA terminals. Poirier and Sourkes (51,52) confirmed this, general tract in the monkeys and cats. They give the origin of the tract as the substantia nigra and parabrachiole pigmentosis and place its position more dorsolaterally than that described by the Swedes and suggest that it may extend more rostrally into the diencephalon. Goldstein (71,72,73) made lesions in the ventrolateral tegmentum of the midbrain and showed decreases in tyrosine hydroxylase activity, in vivo DA synthesis and DA uptake on the ipsilateral side.

It is however, difficult to substantiate these nigro-striatal tracts anatomically. Nauta (63) has described connections from the midbrain to caudate and putamen. Other reports (60,61) indicate that lesions of the substantia nigra cause degeneration only in the red nucleus, superior colliculi and globus pallidus.

The great interest in this nigro-striatal tract originates from its possible connection with Parkinsonism; and experiment have been done with animals in an attempt to relate Parkinsonism, NA and nigrostriatal lesions. In rats, lesions alone, did not produce Parkinson-like symptoms but when reserpine was also administered there was rigidity and tremor on the side contralateral to the lesion. It was postulated that there is a balance between cholinergic

and dopanergic fibers to the striatum. This is reflected at the spinal level in the control of the output of α and γ fibers. When one system (eg. dopanergic) is upset there is hyperexcitability of the α system and reduction in the γ. Poirier (137) was able to produce Parkinson-like symptoms in monkeys with lesions in the nigrostriatal system. Administration of the MAO inhibitor harmaline intensified the motor abnormality but this could not be related to any affect on the nigrostriatal tract. Therefore the exact relationship between DA, serotonin, basal ganglion and Parkinsonism is not yet established.

ii) To the septal area

Less work has been done on the origin and course of the fibers to the septal area. The Swedish workers (6) have postulated that two groups of CA cells (primarily DA) in the midbrain are the origin of fibers to the nucleus accombens and tuberculum olfactorum. One group (designated as A 10) is along the midline just dorsal to the interpeduncular nucleus extending over most of its length; the second group (A8) is just lateral to this at approximately the same level. These workers claim that NA fibers in the septal area originate in the pons and medullary region. Since cerveau-isole lesions (Table 17) produced little change in tyrosine hydroxylase activity in the septal area, we are most likely dealing with fibers originating in the midbrain rostral to the inferior colliculi. Up to 100% of the septal fibers could be accounted for in this region, depending on the lateral extent of the lesion. The fibers and/or the cell bodies to the septal area must therefore be a few mm from the midline but medial to the fibers to the caudate (from the substantia nigra) since it was possible to affect the septal fibers without touching the caudate ones. Whether the origin of the septal fibers in the group A 10 cells with the fibers coursing laterally and rostrally or whether the origin in the A 8 cells with the fibers travelling medially and rostrally is not known.

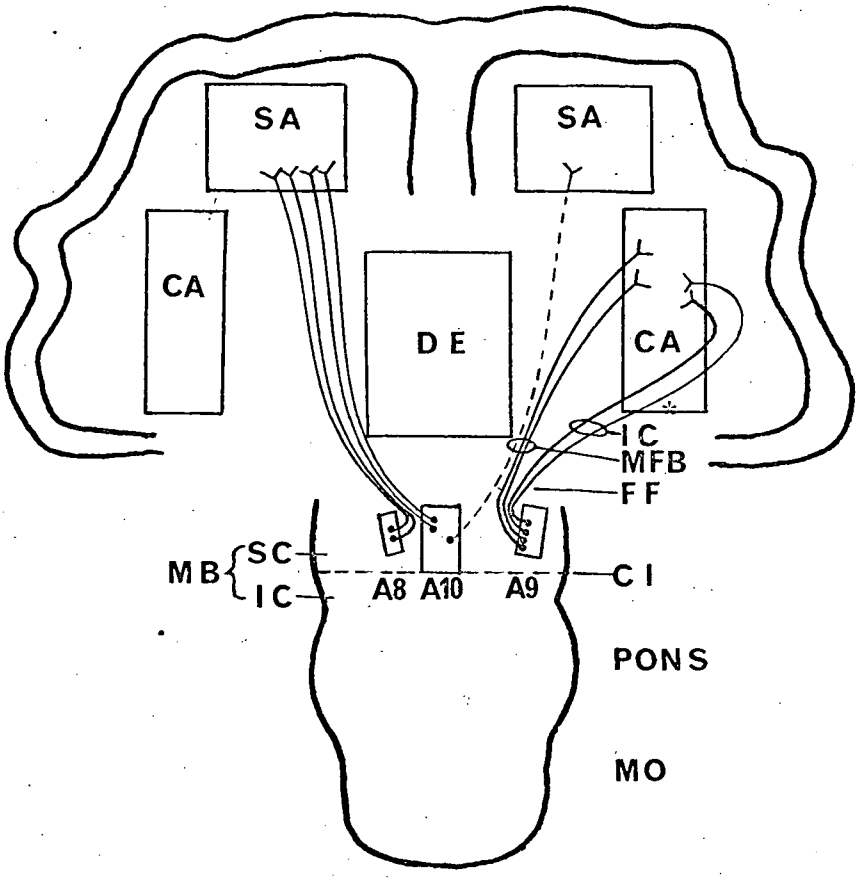
The results obtained could be consistent with either or both explanations.

d) Summary

Diag. 3 is a summary of the possible origins and course of fibers containing tyrosine hydroxylase to the caudate and septal area. The diagram indicates the fibers to the caudate probably originate largely in the substantia nigra, turn medially as they go through the midbrain and posterior diencephalon, where most of them can still be accounted for. The majority of fibers then probably course through the internal capsule to the caudate, though some would appear to course more anteriorly to the mid-diencephalon before entering the caudate, most probably to the medial aspects. The fibers to the septal area could originate in those cells, designated as A 10 and A 8 by the Swedish workers, course medially or laterally respectively as they go through the midbrain slightly medial to the nigrostriatal fibers. In the posterior diencephalon they may be intermingled or dorsal to the fibers to the caudate. However, as the two group of fibers to septal area and caudate course through the diencephalon they begin to separate into medial and lateral positions respectively.

Whether these fibers just described are DA or NA containing is not known. Probably these to the caudate are DA containing. If rats are comparable to cats, it would appear from the Swedish work that the fibers we are dealing with are p primarily dopanminergic, originating in the midbrain and going to the tuberculum olfactorum and nucleus accombens. It is difficult to distinguish NA and DA containing fibers by histochemical techniques, since the only means is different recovery times after. depletion with α-methyl-p-tyrosine. Therefore it cannot be stated at this time whether all cells of the midbrain are primarily DA containing. It is perhaps of interest though that there were no changes in tyrosine hydroxylase activity in the hypothalamus (primarily NA containing fibers) although many other reports indicate a decrease in NA with similar MFB lesion.

Similar lesions studies with the enzyme dopamine β-oxidase could distinguish the NA and DA containing fibers.



Diag 2: Summary of tyrosine hydroxylase containing fibres to the caudate and septal area

SA = septal area
CA = caudate
DE = diencephalon
IC* = internal capsule
MFB = medial forebrain bundle
FF = Fields of Forel
Cl = Cerveau isole
MO = medulla oblongata
MB = midbrain
SC = superior colliculi
IC = inferior colliculi

A 8, A 10, A 9 - refer to groups of CA cells, (as designated by rectangles) in midbrain as described in reference 49, that are possible origins of the fibers in the diagram.

This investigation dealt primarily with determining approximate origins and courses of DA and NA containing fibers without determing exact dorsal, ventral and lateral medial positions, in order to pin point exact nuclei of origin and courses of fibers, more discrete lesions on a larger number of animals are required.

SUMMARY AND CONCLUSIONS

The results of this investigation and possible conclusions that are drawn from these can be summarized as follows:

1) Ion exchange chromatography on Dowex 50 x 8 resin and thin layer chromatography on polyamide have proven to be successful in the separation of NA and DA; their precursors tyrosine and Dopa; and some of their metabolites. Possible applications of these techniques in enzyme analysis and in vivo metabolic studies of CA have been discussed.

2) In conclusive results were obtained in determining the effect of exposure to cold on CA metabolism in rats. Although an increase in CA secretion in urines of animals in the cold was observed, the exact site at which this increase in synthesis takes place was not determined. There was some indication that the adrenal gland may be one organ of increased CA synthesis during cold adaption.

3) The distribution of tyrosine hydroxylase in brain was determined and found to parallel CA concentrations and in vivo determinations of CA synthesis, with highest activity in the caudate, nucleus accombens, septal area and anterior perforating substance. From these distributions studies it would appear tyrosine hydroxylase is a good indication of CA metabolism in various regions of the brain, but it may not be the only control.

4) Fibers containing tyrosine hydroxylase were tentatively traced in the cat from the midbrain (their apparent site of origin) through the diencephalon to the caudate and septal area by means of lesion experimentation. The experiments fibers to the septal area appear to course medially to those of the caudate in the midbrain and mid-diencephalon, but in the posterior diencephalon the two groups of fibers are in close proximity. There were also indications of tyrosine hydroxylase containing fibers to the amygdala and hippocampus in the posterior diencephalon. These results were correlated with findings of other workers studying dopanergic and noradrenergic nerve fibers, and with possible brain

function of these tracts.

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Appendix

DISTRIBUTION OF TYROSINE HYDROXYLASE ACTIVITY IN ADULT AND DEVELOPING BRAIN

E. G. McGeer, S. Gibson, J. A. Wada, and P. L. McGeer
Kinsmen Laboratory of Neurological Research, Faculty of Medicine, The University of British Columbia, Vancouver, B.C.

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The distribution of tyrosine hydroxylase activity in various areas of brain was studied in mature rats, rabbits, cats, and in kittens of various ages. Distribution was closely similar in all species. Areas known to have high concentrations of catecholinergic nerve endings, such as the caudate, septal area and pineal, showed very high adult levels and sharp increases during the neonatal period. Areas such as the pons–medulla and midbrain, known to contain predominantly catecholinergic cell bodies, showed, on the other hand, relatively low adult levels and little or no change in the neonatal period. Developmental data correlate with known neonatal changes in endogenous catecholamine levels and adult distributions correlate with known turnover rates. The results are in conformity with previous findings that tyrosine hydroxylase is concentrated largely in nerve endings and suggest that measurement of this enzymic activity may provide a more convenient and more sensitive index of catecholinergic nerve activity under various conditions than is provided by measurement of the amines themselves.

Tyrosine hydroxylase is the enzyme responsible for conversion of tyrosine to 3,4-dihydroxyphenylalanine (Dopa) (1). This initial step in the biosynthesis of catecholamines is the slow step, and is therefore considered to be rate-limiting for the overall conversion in vivo (2). The enzyme has greatest activity in such tissues as the adrenal medulla and brain, which have high concentrations of catecholamines.

In brain, tyrosine hydroxylase has been shown to be particle-bound, and highly localized in nerve endings (3). This biochemical information ties in closely with histochemical studies showing that noradrenaline and dopamine are also highly localized in nerve endings. Lower concentrations of these amines are found in cell bodies. The concentration of catecholamines in various brain areas, as revealed by histochemical studies, is more or less in accord with biochemical studies showing the distribution of dopamine and noradrenaline (4). However, the turnover rate of radioactive catecholamines is not equal in all brain areas and does not parallel the concentration. For example, the cerebellum has an unusually high turnover rate and yet has a very low concentration of catecholamines (5).

Tyrosine hydroxylase activity should reflect a combination of turnover rate and density of catecholinergic nerve endings in any given region of brain. Consequently, measurement of tyrosine hydroxylase may provide a more sensitive index of catecholinergic nerve activity under various conditions than measurement of the amines themselves.

This paper reports the distribution of tyrosine hydroxylase in various areas of the developing and mature brain. The rat, rabbit, and cat were used for the adult study, and kittens for the developmental study.

TABLE I

Tissue weights of various brain areas*

Area†	Species		
	Rat	Rabbit	Cat
Caudate	33±6	77±7	416±58
Septal area	48±23	70±7	124±26
Pons–medulla	214±30	801±118	1408±163
Midbrain	118±15	536±98	1074±154
Hypothalamus	224±67	115±21	110± 30
Thalamus		400±23	1278±246
Hippocampus	96±26	507±74	582±110
Amygdala	76±14	—	400±62
Cerebellum	258±9	1114±128	3438±201

*Average ± standard deviation in mg.
†Data on weights of cortex and spinal cord are not given since only a portion of these areas was used. For subcortical areas, weights are for tissue pooled from both brain halves.

Materials and Methods

Rats were killed by a sharp blow to the head, rabbits by cervical dislocation, and adult cats by nitrogen asphyxiation. The brains were rapidly removed, and dissected into the areas indicated in Table I and Fig. 1. The tissue was homogenized in 4–19 volumes of ice-cold 0.25 *M* sucrose, the larger volumes of sucrose being used with the more active brain regions. The reproducibility of the dissection procedure is indicated by the data on weights given in Table I.

Kittens of known ages from 2 to 47 days were killed by decapitation. Six litters in all were used: four litters of four kittens each, and two of three. Siblings were always killed at weekly intervals (see Table V for details). The brain was divided as shown in Fig. 1 except that the hypothalamus was pooled

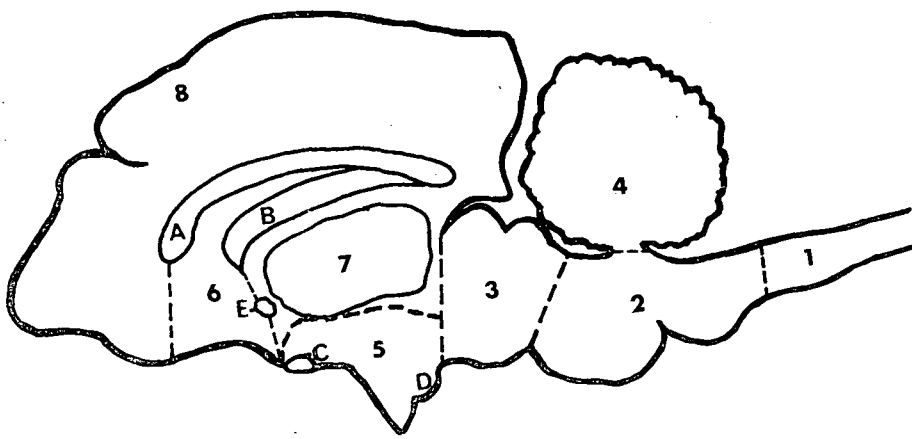

FIG. 1. Sagittal section showing some of areas dissected (numbers) and some landmarks used in dissection (letters) 1 = spinal cord, 2 = pons–medulla, 3 = midbrain, 4 = cerebellum, 5 = hypothalamus, 6 = septal area, 7 = thalamus, 8 = cortex, A = corpus callosum, B = fornix, C = optic chiasma, D = mammillary body, E = anterior commissure.

TABLE II

Endogenous tyrosine levels (Average ± standard deviation in μg/g of tissue)

Area	Adult rat	Adult rabbit	Adult cat	Kitten*
Caudate	28.8±10.2	27.8±5.2	24.6±6.5	22.6±5.7
Septal area	16.5±6.3	24.9±3.5	17.6±6.0	19.4±4.7
Pons–medulla	17.3±3.2	24.1±10.1	13.4±2.8	19.4±3.8
Midbrain	15.6±4.2	25.8±4.2	14.3±7.0	19.1±4.7
Hippocampus	22.7±7.1	28.3±4.0	13.2±3.2	18.4±3.6
Amygdala	23.9±5.1	—	16.1±3.5	
Hypothalamus	20.8±5.4	23.5±5.1	19.8±5.5	20.1±4.2
Thalamus		25.3±6.2	14.5±5.7	
Cortex	15.7±4.5	31.2±4.8	14.8±5.1	20.7±4.5
Cerebellum	17.3±5.5	22.9±8.5	18.9±4.6	23.4±4.2
Spinal cord	22.7±3.5	22.7±3.7	—	—

*No consistent change with age was evident.

with the thalamus and the amygdala was pooled with the hippocampus. Each brain area was homogenized in 5.6 volumes of sucrose. In those cases where pineal activity was measured, the pineal was dissected out and immediately homogenized in 0.3 ml of 0.25 *M* sucrose.

Measurement of tyrosine hydroxylase was done in all cases by the previously reported method (6) in which an aliquot of brain homogenate is incubated with radioactive tyrosine in the presence of a DOPA decarboxylase inhibitor, and the radioactive catecholamines formed are isolated and counted. 2-Amino-4-hydroxy-6,7-dimethyltetrahydropteridine ($DMPH_4$) in combination with mercaptoethanol was not used routinely since this adrenal cofactor system has been repeatedly shown to have no significant effect on rat brain tyrosine hydroxylase activity (6). Similarly, in this study, it was found to have no significant effect on conversions in cat or rabbit brain homogenates. With 11 areas of rabbit brain, for example, conversion with cofactor was 109 ± 17% of that without cofactor, whereas in 18 different brain areas from several adult cats, the conversion was 92 ± 21% of that without. Similar comparative studies with each area in each of four sibling kittens studied at 5–27 days of age again indicated that the $DMPH_4$–mercaptoethanol combination caused no significant enhancement of conversion. The same $DMPH_4$ which had little or no effect on the conversion in various cat brain areas increased the conversion in cat adrenal homogenate more than 10-fold.

All incubations were run at tyrosine concentrations below saturation of the enzyme. The calculation of V_{max} required, therefore, knowledge of the endogenous tissue tyrosine level and of the Michaelis constant for the reaction. Endogenous tyrosine levels (Table II) were determined by a modification of the method of Waalkes and Udenfriend (6, 7).

Repeated determination of Michaelis constants for rabbit brain, for whole cat brain, and for cat caudate yielded an average figure of 1×10^{-5} for K_m in each case. Similar results in all cases were obtained with and without added $DMPH_4$ plus mercaptoethanol. Representative plots used in the determination

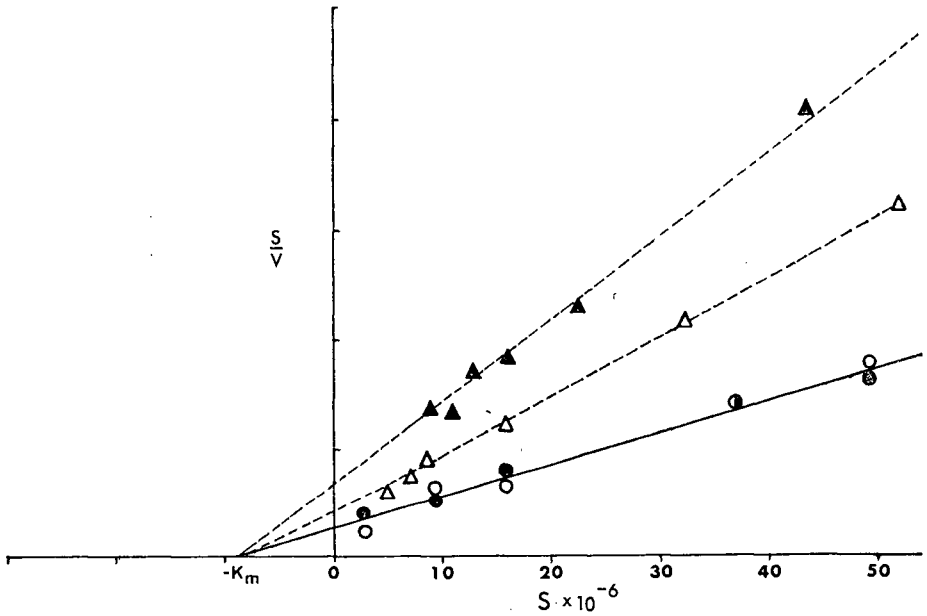

FIG. 2. Sample curves for the determination of the Michaelis constant for rabbit and cat brain. Plots drawn as recommended by Dowd and Riggs (8). Solid symbols with $DMPH_4$ plus mercaptoethanol; empty symbols without. Rabbit data (▲— and △—) obtained on two different tissue samples; cat data (●— and ○—) obtained in parallel analyses on aliquots of the same brain homogenate.

of the Michaelis constant are given in Fig. 2. The previously determined Michaelis constant for whole rat brain homogenate (4.5×10^{-6}) was used in calculation of the rat data (6).

The measured velocity, V_{meas}, could be calculated from the amount of radioactive tyrosine converted to radioactive catecholamines. An overall velocity, V, was related to the measured velocity by the equation:

$$V = V_{meas}\left[\frac{S_E + S_R}{S_R}\right],$$

where S_E = concentration of endogenous tyrosine and S_R = concentration of radioactive tyrosine.

The V_{max} in each case could be calculated from V by the equation:

$$V_{max} = V\left[1 + \frac{K_m}{S_E + S_R}\right].$$

In the developmental study, the tissue homogenate remaining after removal of aliquots for enzyme and tyrosine assay was used in many cases for analysis of endogenous catecholamine levels. For this purpose the volume was measured, and then the homogenate was made 0.2 N in perchloric acid. The further procedure for isolation and determination of the catecholamines was as reported previously for brain tissue homogenates (9).

TABLE III

Distribution of tyrosine hydroxylase activity in adult rat, rabbit, and cat brain*

Area	V_{max} in nmoles DOPA/h/g of tissue			Activity relative to cerebellum		
	Rat	Rabbit	Cat	Rat	Rabbit	Cat
Caudate	72.3±5.5 (6)*	70.9±9.2 (5)	98.5±9 (11)	56	101	70
Septal area	15.0±2.4 (6)	20.3±1.6 (4)	19.0±5 (12)	11.5	29	13.5
Amygdala	4.2±0.5 (3)	—	4.2±1 (11)	3.2	—	3.0
Hypothalamus	5.0±1.6 (6)	3.7±1.0 (5)	3.9±0.7 (11)	3.8	5.3	2.8
Thalamus		0.6±0.1 (5)	2.7±0.2 (9)		0.9	1.9
Midbrain	3.6±1.0 (6)	2.2±0.5 (5)	3.1±0.2 (5)	2.8	3.1	2.2
Pons–medulla	2.9±0.3 (6)	1.7±0.2 (5)	1.8±0.5 (13)	2.2	2.4	1.3
Cortex	3.1±0.8 (6)	2.2±1.0 (5)	2.5±0.6 (9)	2.4	3.1	1.8
Hippocampus	1.8±0.4 (4)	2.1±0.6 (5)	1.6±0.4 (10)	1.4	3.0	1.1
Spinal cord	1.2±0.7 (5)	2.2±0.9 (4)	—	0.9	3.1	—
Cerebellum	1.3±0.4 (6)	0.7±0.2 (4)	1.4±0.2 (4)	1.0	1.0	1.0

*Number of animals given in parentheses.

Results and Discussion

The data obtained on the distribution of tyrosine hydroxylase activity in various areas of adult rat, rabbit, and cat brain are summarized in Table III. It is evident that the distribution follows a similar pattern in all three species, and that tyrosine hydroxylase activity is highly concentrated in the caudate and septal area, regions with extremely high endogenous dopamine levels and very rich in catecholinergic nerve endings (10). The hypothalamus and midbrain, areas rich in noradrenaline but relatively low in dopamine, contain much less tyrosine hydroxylase than does the striatum.

Since tyrosine hydroxylase activity may be expected to correlate with total catecholamine turnover rather than endogenous levels, it is interesting to compare the relative activities found with such estimates of relative turnover as are available. Iversen and Glowinski (5) have estimated noradrenaline turnover rates in five areas of rat brain from data on disappearance rates for injected ^{3}H-noradrenaline and from endogenous noradrenaline levels. Their estimates, expressed as relative to estimated cerebellar turnover, are given in Table IV along with the relative tyrosine hydroxylase activities found for these areas. The agreement, except for the cortex, is remarkably close.

Striatal tyrosine hydroxylase levels might be expected to reflect dopamine rather than noradrenaline turnover. Iversen and Glowinski (5), using two different methods, arrive at a half-life for striatal dopamine of 1.5–4 h, and Glowinski and Iversen (11) report endogenous striatal dopamine levels in the rat of 7.5 μg/g. These data would correspond to a turnover rate of 6–16 nmoles of dopamine per gram per hour, or approximately 23-62 times the turnover rate estimated by these authors for noradrenaline in the cerebellum. This ratio again appears to be of the same order of magnitude as the ratio of striatal tyrosine hydroxylase activity to that of cerebellum.

It must be pointed out that, although the relative data are in general agreement, the absolute conversions indicated by the tyrosine hydroxylase data are

TABLE IV

Relative tyrosine hydroxylase activities and noradrenaline turnover rates in rat brain areas

	Relative tyrosine hydroxylase	Relative NA turnover*
Hypothalamus	>3.8†	5.6
Medulla	2.2	2.1
Cortex	2.4	0.9
Hippocampus	1.4	0.8
Cerebellum	1.0	1.0

*Based on data of Iversen and Glowinski (5).
†Given as > 3.8 because the hypothalamic activity in rats was undoubtedly diluted by the thalamus which has a lower order of activity (see Table III).

five- to sixfold greater than those indicated by the turnover rates. The tyrosine hydroxylase figures given, of course, are for V_{max} under in vitro conditions where feedback inhibition by product amine would be negligible, and where saturation of the enzyme with tyrosine could be assumed. This discrepancy, however, accords with other indications that it is not enzyme capacity but rather enzyme utilization that is the rate-limiting factor of in vivo catecholamine synthesis.

Data obtained in the developmental study generally confirmed the results on distribution. However, there were some significant changes with postnatal age in certain areas which accord with previous data of Pscheidt and Himwich (12). They reported that many regions of cat brain show a progressive increase in serotonin and noradrenaline levels with increasing postnatal age and that each of these regions has a distinctive pattern of accumulation of amines. The data for tyrosine hydroxylase for six areas (caudate, pineal, septal, pons–medulla, cortex, and amygdala–hippocampus) similarly indicated a change in activity with postnatal development. These data are plotted in Fig. 3A. Figure 3B shows corresponding data for endogenous noradrenaline levels. Those data of Pscheidt and Himwich (12) for noradrenaline levels are included in Fig. 3B where the brain area studied seemed comparable. It is evident that there is good agreement between our data for noradrenaline and those of Pscheidt and Himwich, considering the probable differences in dissection. It is also evident that in those areas where sizeable changes in noradrenaline levels occur, similar changes in tyrosine hydroxylase activity occur and often precede the changes in amine level. Thus, the drop-off in noradrenaline levels in the pons–medulla at 30–90 days noted by Pscheidt and Himwich correlates with a fall in tyrosine hydroxylase activity between the 22nd and the 40th day after birth. Similarly the sharp rise in caudate noradrenaline found by Pscheidt and Himwich at about the 80th day is preceded by a sharp increase in tyrosine hydroxylase. A large relative increase in enzyme activity occurs in the cortex between the 8th and 15th days which corresponds to the time when Pscheidt and Himwich noted the beginning of a steady rise in noradrenaline.

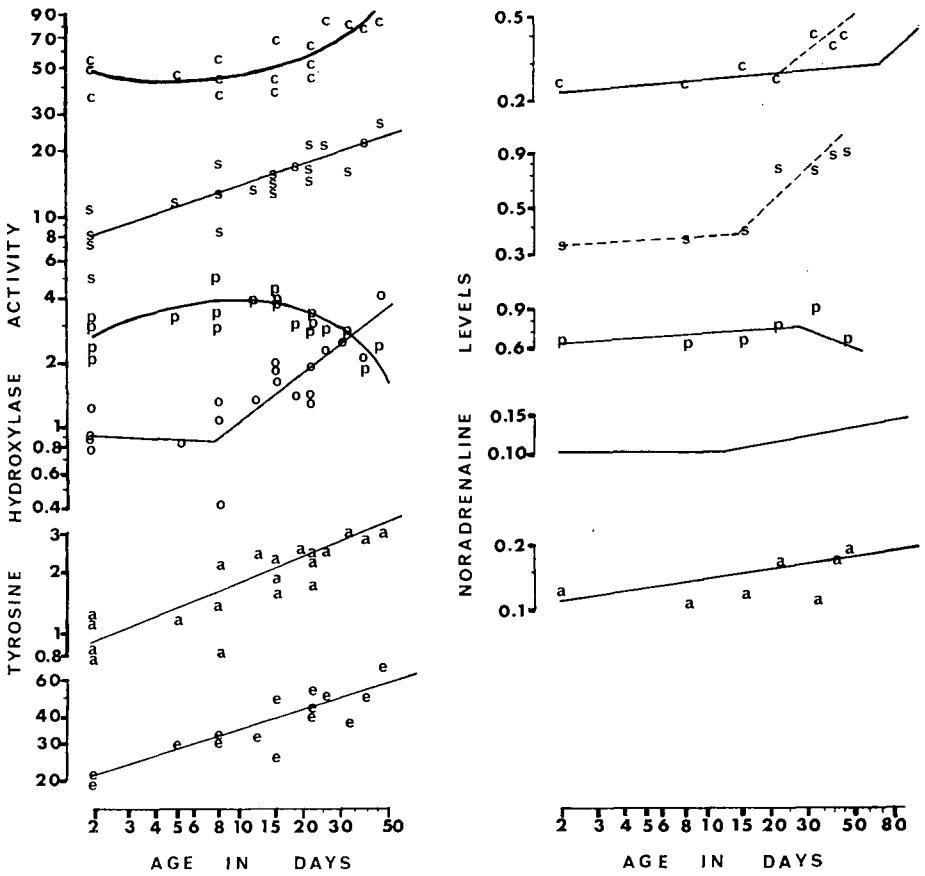

FIG. 3. Tyrosine hydroxylase activity and endogenous noradrenaline levels in some areas of developing cat brain. Log–log scale. Each symbol represents one tissue sample in the case of tyrosine hydroxylase activity and an average value for noradrenaline levels. c = caudate, s = septal area, p = pons–medulla, o = cortex, a = amygdala–hippocampus, e = pineal. Tyrosine hydroxylase activity in terms of V_{max} in nmoles/g tissue/h except for pineal where V_{max} is in pmoles/pineal/h. Noradrenaline levels in μg/g of tissue. Solid lines showing changes in noradrenaline levels represent data of Pscheidt and Himwich (12); symbols and dotted lines show our data. Solid line without any symbols is cortical data of Pscheidt and Himwich.

Data for tyrosine hydroxylase levels for the cerebellum, midbrain, and hypothalamus–thalamus are given in Table V; these data are not plotted since they do not indicate any significant postnatal change.

All the brain areas studied increased in weight as the kittens aged from 2 to 47 days. The cerebellum showed the largest (ninefold) increase and the septal area the smallest (twofold). There was no correlation between changes in weight and in tyrosine hydroxylase activity per unit weight. This is illustrated in Table VI which indicates the weights at various ages of the cortex, caudate, midbrain, and pons–medulla.

TABLE V

Tyrosine hydroxylase activity in cerebellum, midbrain, and hypothalamus–thalamus in neonatal cats*

Age in days	Cerebellum	Midbrain	Hypothalamus –thalamus	Litter designation
2	0.8±0.3	3.3±1.3	3.0±0.7	A,B,C,D†
5	1.0	2.3	4.3	E
8	1.7±0.3	3.3±1.0	3.3±0.6	A,B,C
12	1.1	3.3	3.1	E
15	1.9±0.3	3.9±1.4	3.3±0.9	A,B,C
19	1.1	3.9	2.6	E
22	1.1±0.2	3.0±0.6	2.5±0.8	A,B,C
26	1.3	3.6	2.7	E
33	1.5	4.1	3.2	F
40	0.7	2.3	2.7	F
47	0.9	3.8	3.3	F
Average of above	1.3±0.4	3.4±0.9	3.0±0.7	
Adult level	1.4±0.2	3.1±0.2		

*V_{max} in nmoles of DOPA formed/h/g of tissue.
†Littermates of this kitten were lost.

TABLE VI

Tissue weights of selected brain areas in neonatal cats

Age in days	Caudate	Cortex	Midbrain	Pons–medulla
2	101±21	3070±330	272±56	242±48
5	88	3620	300	270
8	149±62	5760±220	316±62	372±89
12	156	6090	370	432
15	203±44	7818±348	408±17	409±126
19	455*	8410	500	443
22	260±20	10160±330	482±64	504±39
25	328	10600	530	658
33	281	12600	592	720
40	307	12060	665	803
47	367	13874	672	875

*Data on this sample are not included in Figs. 3 or 4 since the weight indicates a major variation in dissection.

In all the kittens studied, as well as in the adult cats, the caudate was by far the most active area in terms of tyrosine hydroxylation per gram of tissue. When the weight of tissue is considered, however, it is evident that, in the older cats, the cortex, despite its lower unit activity, makes a greater contribution to total tyrosine hydroxylase activity, and presumably also to catecholamine synthesis, than does any other brain area including the caudate. The caudate contribution is 30–45% of the total tyrosine hydroxylase activity of brain, with the higher figures tending to occur in the younger kittens. The percentage contribution of the cortex rises from a figure of less than 30% in the 2-day-old kittens to more than 40% in the older kittens. The time at which the cortical contribution becomes greater than the caudate is about 12 days (Fig. 4), which is coincident with the first signs of maturation of the EEG in kittens (12).

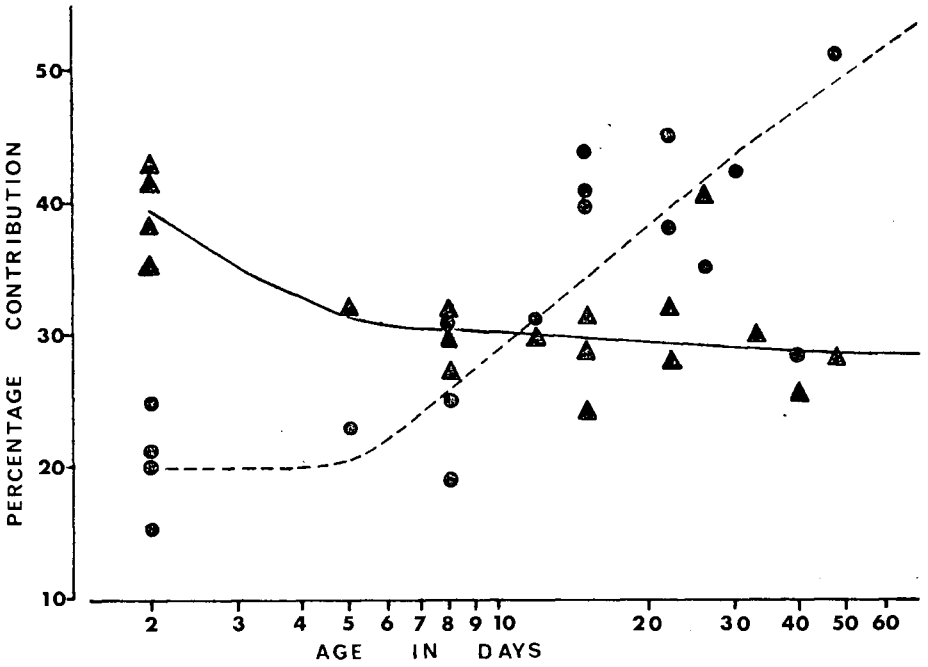

FIG. 4. Percentage contribution of caudate and cortex to total brain tyrosine hydroxylase activity in kittens. Semilog scale. Circles and dashed line for cortex; triangles and solid line for caudate.

The pons-medulla and midbrain, where catecholinergic cell bodies are almost entirely concentrated (10, 13), showed no appreciable rise in tyrosine hydroxylase activity during postnatal development. The caudate, the limbic structures, and the cortex, on the other hand, which contain the terminal nerve endings of these cell bodies, showed sharp increases in tyrosine hydroxylase activity. The pineal gland, which contains sympathetic nerve endings but not cell bodies (14), also showed a sharp increase with age.

Tyrosine hydroxylase is known to be concentrated in nerve ending particles (3). Presumably the postnatal increases in tyrosine hydroxylase activity reflect growth and development of catecholinergic nerve endings, and the marked differences in activity per gram of tissue found in the various adult brain areas reflect the wide variation in concentration of such nerve endings. Both types of data are in general accord with such data as have so far been accumulated through histochemical studies (4, 10, 13).

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Appendix

SOME CHARACTERISTICS OF BRAIN TYROSINE HYDROXYLASE

E. G. McGeer, S. Gibson, and P. L. McGeer

Kinsmen Laboratory of Neurological Research, Faculty of Medicine, The University of British Columbia, Vancouver, British Columbia

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Tyrosine hydroxylase from brain homogenates differed from tyrosine hydroxylase from adrenal homogenates in being particle-bound, insensitive to cofactors, possessing a lower Michaelis constant for tyrosine, and being responsive to slightly different optimum conditions of pH and buffer. The combination of 0.02 *M* mercaptoethanol and 0.1–1.0 m*M* 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine ($DMPH_4$) increased tyrosine hydroxylase activity in beef adrenal homogenates 15-fold, but was without effect on activity in rat brain homogenates. The K_m for tyrosine in beef adrenal homogenates was 4×10^{-5} *M*, and in rat brain homogenates was 0.45×10^{-5} *M*. Conversion in beef adrenal homogenates was maximum in 0.6 *M* sodium acetate buffer, pH 6.0, and in rat brain homogenates was maximum in 0.28 *M* phosphate buffer, pH 6.2.

Introduction

Much interest has been aroused in tyrosine hydroxylase, the enzyme which converts tyrosine to 3,4-dihydroxyphenylalanine (DOPA), because it appears to be the rate-limiting enzymatic step in catecholamine synthesis (1). Kinetic studies have been done chiefly with the enzyme from adrenal medulla (2–7). The enzyme from brain seems to differ in some respects from the enzyme from the adrenal medulla (2–4). This paper reports some further kinetic data on brain tyrosine hydroxylase and gives the basis for the assay method used in this laboratory.

Materials and Methods

Tissue was rapidly removed from animals sacrificed by a sharp blow to the neck. It was weighed and homogenized in 4–9 volumes of ice-cold 0.25 *M* sucrose. A 0.1-ml aliquot was incubated in an air atmosphere with 0.1 ml of a radioactive tyrosine solution and 0.1 ml of buffer. The buffer was either 0.28 *M* phosphate, pH 6.2 (preferred for brain), or 0.6 *M* acetate, pH 6.0 (preferred for adrenal). The buffer was often made 3×10^{-3} or 10^{-4} *M* in 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine ($DMPH_4$) and 6×10^{-2} *M* in 2-mercaptoethanol. Typically the radioactive tyrosine solution contained 140,000–280,000 c.p.m. (1/6–1/12 mCi) of L-tyrosine-^{14}C (uniformly labelled; specific activity 330 mCi/mmole) and was made 3×10^{-3} *M* in *N*-methyl-*N*-3-hydroxyphenylhydrazine (NSD-1034), a DOPA decarboxylase inhibitor. Blanks were run by incubating with tyrosine-^{14}C tissue homogenates, initially heated to 80–90 °C for 12–15 min and then cooled in ice. Incubations at 37 °C were generally 30 min for brain homogenates and 10 min for adrenal homogenates. The reactions were stopped by the addition of 2 ml of a 1:1 mixture of 0.4 *N* perchloric acid and 0.2 *N* acetic acid, containing cold carrier

DOPA, dopamine, and noradrenaline (0.2 μg of each). The mixtures were frozen until work-up.

The $DMPH_4$ used was obtained from Calbiochem, and the NSD-1034 from Dr. D. J. Drain of Smith and Nephow Research.

If $DMPH_4$ was to be employed, an aqueous solution 10^{-2} or 10^{-3} M in $DMPH_4$ and 0.2 M in 2-mercaptoethanol was prepared just prior to the experiment. Such a solution retained activity for several hours if kept on ice in the dark. For incubations, a 0.3-ml aliquot of this solution was mixed with 0.7 ml of either 0.4 M phosphate buffer, pH 6.2, or 0.85 M acetate buffer, pH 6.0; 0.1 ml of the mixture was used immediately in each incubation.

Since the tyrosine hydroxylase incubation is run at substrate concentrations below saturation, knowledge of endogenous tyrosine levels is necessary for calculation of V_{max}. A small aliquot of the homogenate was mixed with an equal volume of 30% trichloracetic acid and three volumes of water, and the supernate was used for analysis of endogenous tyrosine by the method of Waalkes and Udenfriend (8) modified only in the use of 0.6 ml each of supernate, 1-nitroso-2-naphthol and dilute nitric acid, and 2.5 ml of ethylene dichloride.

Isolation and measurement of the radioactive catechols was on an alumina column. The incubation mixture was thawed and centrifuged briefly. The clear supernate was poured into a 20-ml beaker containing 1.25 ml of 0.2 M ethylenediaminetetracetic acid (EDTA). The contents of the incubation tube were rinsed into the centrifuge tube with 1.5 ml of 0.35 M KH_2PO_4 followed by a little water. The mixture was centrifuged again and the clear supernate combined with the first supernate plus EDTA. The solution was then taken to pH 8.8–9.2 with dilute sodium hydroxide, approximately 350 mg of acid-washed alumina was added immediately, and the mixture was stirred for 4–5 min on a magnetic stirrer. It was then poured into a glass column plugged with cellulose fiber. A slight vacuum was used to promote rapid passage of the solution through the column. The alumina was washed from the beaker onto the column with approximately 20 ml of water. This and a second 20 ml wash were passed through the column under a slight vacuum. The column was then removed from the vacuum flask and eluted immediately with 2 ml of 0.5 N acetic acid; the eluate was collected in a scintillation vial and was counted in a liquid-scintillation spectrophotometer after addition of 10 ml of Bray's mixture (9). The whole process from the time of making the incubation mixture alkaline to beginning of the acid elution occupied about 10 min but never longer than 15 min. The incubation mixtures were occasionally diluted further (to about 15–20 ml) before making alkaline and stirring with alumina; the results were closely comparable so long as the amounts of phosphate and EDTA were increased accordingly.

The pH used to put the catechols upon the alumina column was chosen after experiments with this particular lot of alumina and with small amounts of radioactive DOPA added to mock-incubation mixtures. Under the conditions specified, the recovery of radioactive DOPA was consistently 65–70%. In the calculations, a recovery of 65% was assumed.

TABLE I

Effect of phosphate on alumina-column absorption of radioactive products

	c.p.m. recovered from column with 6–8 ml of solution*		
	No phosphate	1.5 ml of 0.35 *M* KH_2PO_4	3 ml of 0.35 *M* KH_2PO_4
Freshly prepared tyrosine solution (140,000 c.p.m.; 0.05 μg)	370	192–217	206
Tyrosine frozen 50 days in solution (140,000 c.p.m.; 0.05 μg)	1,651–2,358	247	259
DOPA (2,150 c.p.m.; 0.11 μg)	1,425	1,460	1,410
DOPA (6,450 c.p.m.; 0.33 μg)	4,302	4,209	4,298

*The solution in each case was a mock-incubation mixture and contained 1 ml 0.4 *N* perchloric acid, 1 ml 0.2 *N* acetic acid, 1.25 ml 0.2 *M* EDTA, 0.1 ml 0.25 *M* sucrose, 0.1 ml 0.28 *M* phosphate, 0.2 μg each of cold tyrosine, DOPA, dopamine, noradrenaline, and water to make up to volume. The cold DOPA was decreased to 0.1 μg in the solution with 2,150 c.p.m. DOPA-^{14}C, and omitted from the solution with 6,450 c.p.m. DOPA-^{14}C.

Results

The blanks were typically 190–250 c.p.m. when 140,000 c.p.m. of tyrosine-^{14}C were used, and 350–425 when 280,000 c.p.m. were used. Tests varied from about 600 to 30,000 depending on the tissue used. Heated tissue from various brain areas or from the adrenal always gave the same blank. Incubations of test and blank were always run in triplicate for the first sixty or so experiments. Agreement was invariably within a few percent, so that only duplicates were usually run in the later experiments.

An important modification to the normal procedure for isolating catechols was the use of phosphate in the mixture to be put on the column. Radioactive tyrosine which has been in solution for some time contains as much as 1-2% of impurity which may be absorbed on the alumina column. Phosphate appears to prevent or minimize the absorption of these impurities without affecting the absorption of DOPA (Table I). Purification of the radioactive tyrosine each time before use involves tedious procedures. In practice, it proved more satisfactory to use phosphate in the absorption mixture and to prepare dilutions of the radioactive tyrosine in small quantities which were rapidly used.

The volume of sucrose used for homogenation was chosen in order to yield 5–20 mg of tissue per incubation; generally 15–20 mg were used. With all but the most active brain regions, 5 mg tended to give counts which were lower than desirable for accuracy; above 20 mg of tissue, the conversion tended to fall off. A typical experiment with a rat brain homogenate using 5, 10, 15, and 20 mg of tissue gave calculated $V_{max}(s)$ of, respectively, 22.6, 25.6, 25.4, and 24.9 nmoles per hour per gram of tissue. A typical experiment with a beef adrenal homogenate using 2.5, 10, 15, and 20 mg of tissue gave calculated $V_{max}(s)$ of, respectively, 1201, 1090, 1180, and 1120 nmoles per hour per gram of tissue.

Repeated tests with rat brain homogenate indicated that the reaction was linear at least up to 40 min. Thirty minutes was chosen as the usual incubation time. A few experiments with beef adrenal homogenate suggested that the period of linearity was shorter than with brain homogenate and a 10-min time of incubation seemed preferable.

TABLE II

Relative tyrosine hydroxylase activities of rat brain, cat brain, and beef adrenal homogenates in various buffers*

Buffer	Relative activity, %		
	Rat	Cat	Beef
0.28 *M* K_3PO_4, pH 6.2†	100	100	100
0.28 *M* Na_3PO_4, pH 6.2	101	—	98
0.6 *M* KAc, pH 6.2	48	59	127
0.6 *M* NaAc, pH 6.2	64.5	74	124
0.6 *M* NaAc, pH 6.0	41	55	132
0.6 *M* Tris, pH 6.2	61	51	—

*Data cited were obtained with 10^{-3} *M* $DMPH_4$ and 0.02 *M* 2-mercaptoethanol; comparable relative results were obtained in each series without these additives.
†Chosen as reference conditions.

The choice of phosphate buffer for brain homogenates and of acetate buffer for adrenal homogenates was based on comparative experiments illustrated in Table II. Acetate buffer has been used by others for adrenal tyrosine hydroxylase (2, 3, 5) and there appears to be little pH change during the incubation. Beef caudate resembled rat and cat brain rather than beef adrenal in that the activity in 0.6 *M* acetate buffer, pH 6.0, was 60% of that in 0.28 *M* phosphate buffer, pH 6.2.

Figure 1 shows the effect of buffer pH on tyrosine hydroxylase activity in adrenal (Fig. 1*a*) and rat brain (Fig. 1*b*) homogenates, and Table III indicates the effect of some changes in molarity of sucrose and phosphate on the activity of a rat brain homogenate.

Addition of a variety of ions at 10^{-4} *M* final concentration (Zn^{2+}, Fe^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+}, Co^{2+}, or Fe^{3+}) failed to have any significant effect on enzyme activity in rat brain homogenates either in the presence or absence of $DMPH_4$. Ferrous ion has been reported to stimulate the activity in beef adrenal to a small extent itself, markedly in the presence of tetrahydrofolate and insignificantly in the presence of $DMPH_4$ (2). Addition of 10^{-4} *M* Fe^{2+} ion to a beef

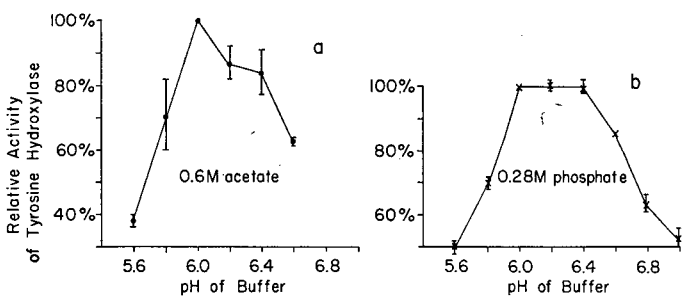

FIG. 1. Effect of buffer pH on tyrosine hydroxylase activities in beef adrenal (*a*) and rat brain (*b*) homogenates. Graphs are based on three series for rat brain and two for adrenal homogenates. In each series, activity at each given pH was expressed as a percentage of that found at pH 6.0. Range of data is indicated by lines.

TABLE III

Relative tyrosine hydroxylase activities of a rat brain homogenate using different molarities of phosphate and sucrose

Phosphate buffer, pH 6.2	Sucrose			
	0.2 *M*	0.25 *M*	0.28 *M*	0.30 *M*
0.2 *M*	22%	44%	17%	39%
0.24 *M*	41%	48%	54%	50%
0.28 *M*	24%	100%*	89%	72%
0.32 *M*	37%	96%	104%	78%
0.35 *M*	20%	85%	93%	64%

*Chosen as reference conditions.

adrenal incubation in the presence of 10^{-4} *M* $DMPH_4$ gave an activity 127% of that in its absence. Mg^{2+}, Ca^{2+}, and Fe^{3+} had no effect whatsoever.

Flushing the incubation tubes with oxygen did not significantly increase observed conversions, but flushing with nitrogen caused very large decreases.

The most significant difference in tyrosine hydroxylase activity between rat brain and beef adrenal homogenates is the dependence of the latter on $DMPH_4$ and 2-mercaptoethanol. These cofactors in combination did not significantly affect activity in crude rat brain homogenates, although they increased adrenal homogenate activity 15-fold. The data in Table IV illustrate not only this effect, but also the inhibitory effect of 2-mercaptoethanol by itself. This inhibition is not surprising in view of the inhibitory effect found for related compounds such as ethanol and L-cysteine (10). $DMPH_4$ by itself increased the activity of adrenal homogenates roughly threefold, but had no effect on brain homogenates.

In compiling the data shown in all tables, a separate blank was run for each test condition. The blanks were generally unaffected by variations in pH, molarity, or buffer type but tended to be 50–60% higher in the presence of $DMPH_4$ than in its absence.

TABLE IV

Relative tyrosine hydroxylase activities in the presence of various additives

Additive (final concentration)	Relative activity, %	
	Beef adrenal (acetate buffer)	Rat brain (phosphate buffer)
None*	100	100
10^{-3} *M* $DMPH_4$ plus 0.02 *M* 2-mercaptoethanol (SH)	1520	92
10^{-3} *M* $DMPH_4$ plus 0.02 *M* SH, preincubated for 10 min	—	75
10^{-4} *M* $DMPH_4$ plus 0.02 *M* SH	1495	96
10^{-3} *M* $DMPH_4$	280	98
0.02 *M* SH	89	79
10^{-3} *M* TPNH	—	97
10^{-3} *M* folic acid	—	98

*Chosen as reference condition.

TABLE V

K_m ($\times 10^{-5}$) for tyrosine determined with and without added $DMPH_4$ (0.001 *M*) plus 2-mercaptoethanol (0.02 *M*)

	With cofactor	Without cofactor	No. of runs
Rat brain	0.45±0.08	0.47±0.05	7
Beef adrenal	4.0±1.2	nonlinear	4

In another approach to the cofactor problem, 36 individual rat brain homogenates were run with and without the combination of $DMPH_4$ plus 2-mercaptoethanol under the usual conditions of relatively low tyrosine concentration. The conversion in the presence of $DMPH_4$ was 104 ± 9% of that in its absence.

It appears possible that the effectiveness of exogenous $DMPH_4$ might be related to the length of time between sacrifice of the animal and incubation. The brain studies were done with animals sacrificed in the laboratory and the usual time between sacrifice and assay was generally less than 2 h. The beef adrenal was obtained from the slaughter house and kept on ice during its transport to the laboratory. There were generally about 4–5 h between dissection and assay. Trial of 12 rat brain homogenates allowed to stand in ice for 5 h between the initial assay and reassay, however, indicated no change in activity during such a period of standing, and no significant effect of exogenous $DMPH_4$ plus 2-mercaptoethanol at either time. The conversions with $DMPH_4$ in this series were 96 ± 8% of the conversions without.

Different K_m values were obtained for rat brain and beef adrenal homogenates (Table V), in confirmation of previous reports (3, 4). The value of 4.0 (±1.2) $\times 10^{-5}$ *M* for beef adrenal homogenates is somewhat lower, but in reasonable agreement with the value of 1×10^{-4} *M* for purified adrenal tyrosine hydroxylase reported by Ikeda *et al.* (5). A nonlinear reciprocal plot of conversion versus tyrosine concentration was obtained for adrenal homogenates in the absence of added cofactor, making it impossible to obtain a K_m value for such conditions. At higher tyrosine concentrations, conversion rapidly fell off.

For crude rat brain homogenates, however, identical reciprocal plots were obtained with and without added cofactor. The K_m value obtained, 0.45 (±0.08) $\times 10^{-5}$ *M*, was significantly lower than that for adrenal homogenates.

Discussion

Although brain tyrosine hydroxylase appears to be different from adrenal tyrosine hydroxylase in being particle-bound, insensitive to cofactors, responsive to slightly different conditions of pH and buffer, and possessing a lower Michaelis constant for tyrosine, firm conclusions regarding differences cannot be drawn from crude enzyme preparations.

Attempts to solubilize brain tyrosine hydroxylase as a preparatory step to

purifying it have led, in our hands, to loss of activity. The enzyme sediments with the crude mitochondrial fraction, and upon further separation is shown to be concentrated in the nerve-ending particles (11). Disruption of the nerve-ending particles by osmotic shock and other means has always led to disappearance of tyrosine hydroxylase activity. The data presented here on brain tyrosine hydroxylase may apply directly to the enzyme. But it is equally possible that they apply to the enzyme plus a particle to which the enzyme is attached or within which it is contained. Such a particle could contain essential cofactors which would not be diluted by the homogenization process. This would explain the insensitivity of brain tyrosine hydroxylase to added cofactors. The particle could also limit the availability of tyrosine to the enzyme, and thus account for the lower K_m for tyrosine in brain compared with that in adrenal.

Although brain tyrosine hydroxylase is highly localized to nerve endings, histochemical studies (12) following lesions to catecholamine axons have shown accumulation of catecholamines proximal to the lesion and disappearance of them at the nerve endings. This result is consistent with the concept of continuous transport of tyrosine hydroxylase from the cell body to the nerve terminal, but the nature of the transport poses problems. If the enzyme were free at the transport stage, some soluble tyrosine hydroxylase should be found following homogenization.

Clearly, the true nature of brain tyrosine hydroxylase must await methods which will lead to its purification and which will explain its association with brain particles.

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