INTERACTION OF CENTRAL NEUROTRANSMITTERS IN THE REGULATION OF ADRENAL DOPAMINE BETA-HYDROXYLASE

by

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To Ana and Katerina, wishes and hopes

ABSTRACT

This work concerns the central regulation of dopamine beta-hydroxylase in the adrenal gland of the rat. This enzyme is neurally induced by subjecting rats to stress or to the injection of reserpine. Several neurotransmitter agonists and antagonists were administered in order to clarify the nervous pathways to this organ. The combination of the central or systemic administration of a catecholamine depletor and a serotonin depletor also increases the activity of this enzyme. Serotonin antagonists but not catecholamine antagonists potentiate the action of reserpine. However, the depletion of catecholamines or serotonin alone does not elevate the activity of this adrenal enzyme. Serotonin agonists block the effect of reserpine. Adrenal phenylethanolamine N-methyltransferase exhibits a similar regulation. Electrolytic lesions and administration of a serotonin neurotoxin in the raphe area show that the medial raphe nucleus is the centre of the inhibitory serotonergic pathway regulating adrenal functions. The muscarinic agonist oxotremorine produces a centrally mediated induction of adrenal dopamine beta-hydroxylase. Depletion of central or peripheral catecholamines and serotonin does not modify the elevation of this enzyme activity by oxotremorine. Central and systemic administration of agonists binding to gamma-aminobutyric acid-receptors decreases adrenal dopamine beta-hydroxylase activity and impairs the effect of the cholinergic inducer. These negative effects are blocked by a specific antagonist of gamma-aminobutyric acid type A-receptors. This work suggests a central inhibitory action of the aminoacid and a gamma-aminobutyric-cholinergic interaction in regard to adrenal dopamine beta-hydroxylase. Corticotropin releasing factor, centrally

administered at a constant rate, increases adrenal dopamine beta-hydroxylase and phenylethanolamine N-methyltranferase activities without elevating plasma corticosterone. This result supports the role of the peptide as first mediator of the stress response.

RESUME

Ce travail porte sur les mécanismes centraux contrôlant l'activité de la dopamine beta-hydroxylase dans la glande surrénale du rat. Cette enzyme est induite par voie nerveuse lorsque les rats sont soumis à un stress ou lorsqu'on leur injecte de la réserpine. Plusieurs substances agonistes et antagonistes des neurotransmitteurs furent administrées afin de déterminer les voies nerveuses contrôlant la fonction de la glande surrénale. L'administration combinée, centrale ou périphérique, de substances causant de fortes diminutions de catécholamines et de substances diminuant le niveau de sérotonine augmente également l'activité de l'enzyme. Les antagonistes de la sérotonine, contrairement aux antagonistes des catécholamines agissent, en synergie, avec la réserpine. Par contre, une réduction des catécholamines ou de la sérotonine uniquement ne produit pas d'augmentation de l'activité enzymatique. Les agonistes de la sérotonine bloquent l'effet de la reserpine. La phényléthanolamine N-méthyltransférase surrénalienne est contrôlée de façon similaire. Le rôle du noyau du raphé médian en tant qu'origine de la voie nerveuse inhibitrice de la fonction surrénalienne a été démontré à l'aide de lésions électrolytiques ou d'injections d'une neurotoxine dans la région du raphé. L'oxotrémorine, un agoniste muscarinique, induit la dopamine beta-hydroxylase via un mécanisme central. Une réduction centrale ou périphérique des catécholamines et de la sérotonine n'a aucun effet sur l'augmentation d'activité enzymatique produite par l'oxotrémorine. L'administration, qu'elle soit centrale ou périphérique, de substances agonistes se liant au récepteur de l'acide gamma-aminobutyrique réduit l'activité de la dopamine beta-hydroxylase, et diminue l'effet de l'inducteur cholinergique. Ces

effets négatifs sont bloqués par l'injection d'un antagoniste spécifique aux récepteurs gabaergiques de type A. Ces études suggèrent un rôle central inhibiteur pour cet acide aminé et une interaction gaba-acétylcholine en ce que concerne le contrôle de l'activité de la dopamine beta-hydroxylase. La corticolibérine, administrée au niveau central et à taux constant augmente l'activité de la dopamine beta-hydroxylase et celle de la phényléthanolamine N-méthyltransférase sans toutefois élever la corticostérone plasmatique. Ce résultat plaide en faveur d'un rôle pour le peptide en tant que premier médiateur de la réponse au stress.

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LIST OF PUBLICATIONS

In accordance with the Guidelines Concerning Thesis Preparation the Candidate has chosen the option of including as part of her thesis the text of original papers already submitted or suitable for submission to learned journals for publication. This thesis conforms to other requirements explained in the Guidelines Concerning Thesis Preparation, McGill University, Faculty of Graduate Studies and Research, revised April, 1984.

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ABBREVIATIONS

ACh	Acetylcholine
ACTH	Adrenocorticotropin
AD	Adrenaline
AMPT	alpha-Methyl-p-tyrosine
ANOVA	Analysis of variance
CA	Catecholamines
cAMP	Cyclic adenosine-3',5'-monophosphate
cDNA	Complementary deoxyribonucleic acid
CNS	Central nervous system
cpm	Counts per minute
CRF	Corticotropin releasing factor
d	Day
DA	Dopamine
DBH	Dopamine beta-hydroxylase (EC 1.14.2.1)
DDC	3,4-Dihydroxyphenylalanine decarboxylase (EC 4.1.1.26)
DHT	5,7-Dihydroxytryptamine
DOPA	3,4-Dihydroxyphenylalanine
DRN	Dorsal raphe nucleus
EDTA	Disodium ethylendiaminetetra-acetate
g	Gram
GABA	gamma-Aminobutyric acid
h	Hour
HPLC	High performance liquid chromatography
5HT	5-Hydroxytryptamine (seorotonin)
icv	Intracerebroventricular
ip	Intraperitoneal

IU	International units
iv	Intravenous
kg	Kilogram
Km	Michaelis Menten constant
M	Molar
uCi	Microcurie
ul	Microliter
umol	Micromol
mCi	Millicurie
ml	Milliliter
mM	Millimolar
MRN	Medial raphe nucleus
PNMT	Phenylethanolamine N-methyltransferase (EC 2.1.1.28)
SAM	S-Adenosylmethionine
sc	Subcutaneous
TH	Tyrosine hydroxylase (EC 1.14.16.2)

1.- INTRODUCTION

1.A. Dopamine beta-hydroxylase

Dopamine beta-hydroxylase (DBH, EC 1.14.2.1) is a mixed function oxidase that catalyzes the conversion of dopamine (DA) to noradrenaline (NA), according to the stoichiometric reaction (Levin et al., 1960):

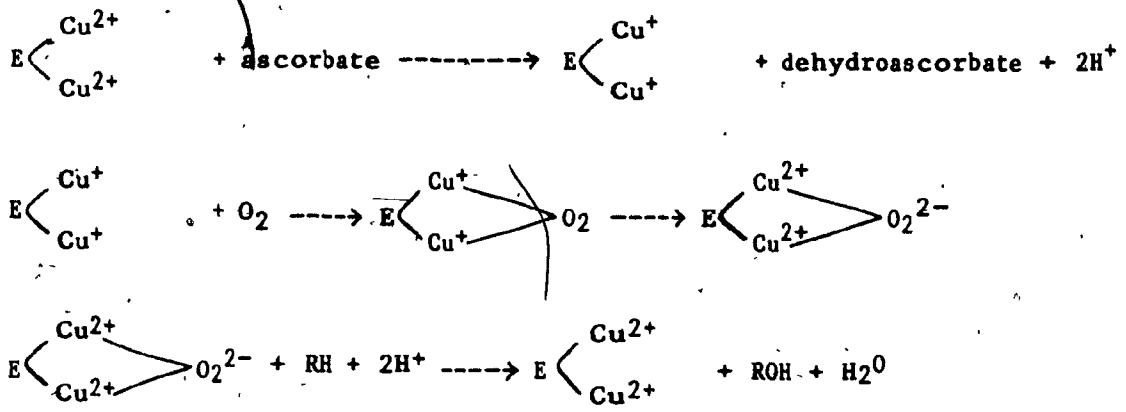
DA + ascorbate + O_2 --------> NA + dehydroascorbate + H_2O

This enzyme has been shown to be relatively nonspecific; it is able to catalyze the hydroxylation of the side chain of many phenylethylamine analogues in the beta position (Goldstein and Contrera, 1962; Levin and Kaufman, 1961). Early studies demonstrated that this enzyme is inhibited by many metal-chelating agents (Levin et al., 1960; Goldstein, 1962; Kirshner, 1962). Later it was proved that the metal associated with DBH is copper (Friedman and Kaufman, 1965), and that this undergoes reduction and oxidation during the enzyme-catalyzed hydroxylation reaction (Friedman and Kaufman, 1965; Blackburn et al., 1980) It was the second mammalian hydroxylase to be shown to contain copper; the only other one known at the time was the phenolase complex (Lerner et al., 1950). Other copper enzymes are: tyrosinase (Vanneste and Zuberbühler, 1974), galactose oxidase (Bereman et al., 1977), cytochrome oxidase (Nicholls and Chance, 1974), superoxide dismutase (Hassan, 1980).

1.A.1. Copper content and mechanism of reaction

An indication that the purified enzyme contained copper was first provided by the observation that a yellow color developed when a concentrated solution of the enzyme was reacted with diethyldithiocarbamate (Friedman and Kaufman, 1965); the spectrum of the treated enzyme was identical to that of the copper diethyldithiocarbamate complex (Friedman and Kaufman, 1965). The

content of copper, unlike that of iron, increased with the purification of the enzyme, which indicates the relation between copper and the enzyme molecule. The purest preparation contains 0.65–1 µg per mg of protein (4-7 moles of copper per mol of enzyme). The enzyme is strongly inhibited by various copper-chelating agents (Goldstein et al., 1965; Friedman and Kaufman, 1965; Skotland and Ljones, 1979), and the reactions with CN^- (Friedman and Kaufman, 1965), bathocuproine disulfonate or EDTA (Skotland and Ljones, 1979) have been used to remove the enzyme-bound copper. The addition of cupric ions ($CuSO_4$) to the inactive apoenzyme (cyanide-treated, for example), restored enzyme activity (Friedman and Kaufman, 1965; Skotland and Ljones, 1979), and the extent of the restoration was a function of the concentration of copper added. However, the maximal recovery of activity was 40% and excess of copper caused inhibition. Experiments in vivo show that DBH activity is decreased in rats made copper deficient by dietary means and that activity is restored by simple addition of $CuSO_4$ to the deficient diet (Missala et al., 1967). It has been proposed that the enzyme-bound copper is involved in electron transfer during the catalytic cycle, because ascorbate can reduce this copper (Friedman and Kaufman, 1965, 1966; Ljones et al., 1978; Diliberto, 1981), which is then reoxidized in the presence of the substrate. This mechanism was formulated by Craine and coworkers (Craine et al., 1973) as follows:



Kinetics experiments support this mechanism (Goldstein et al., 1968).

1.A.2. Activators

Fumarate and other dicarboxylic acids stimulate the rate of hydroxylation by DBH *in vitro* (Levin et al., 1960). Acetate at high concentration can replace fumarate (Goldstein et al., 1968). This stimulation was reported to be due to a lowering of the Km for DA in the presence of atmospheric oxygen at pH 6.4; at pH 6.8 and 7.2, Vmax is also affected (Goldstein et al., 1968). Tris-HCl, sodium phosphate, $NaClO_4$, guanidine HCl (at low concentration), NH_4^+ and NaCl all activate the hydroxylase (Craine et al., 1973). In the absence of activators the optimum pH is 6.4, whereas in their presence it is shifted to the right (7.0-7.1). High concentration of substrate also shifts the pH optimum downward, suggesting the dissociation of an ionizable group on the enzyme-substrate complex (Craine et al., 1973). Craine and his colleagues (Craine et al., 1973) have also shown by measurement of circular dichroism that fumarate and succinate have no effect on the conformation of DBH. Electron spin resonance data show that fumarate increases the Cu^{2+} signal of the enzyme (Friedman and Kaufman, 1966); this could be by a fumarate-induced change in the conformation of the enzyme or a fumarate-dependent change in the ligand field surrounding the enzyme-bound copper (Craine et al., 1973).

1.A.3. Structure

DBH is contained in the chromaffin cells of adrenal medulla (Kirshner, 1957), in peripheral and central adrenergic neurons (Potter and Axelrod, 1963) and in a soluble form in blood plasma (Weinshilboum and Axelrod, 1970).

The enzyme has been purified from bovine, sheep and rat adrenal glands (Foldes et al., 1972; Rush and Geffen, 1972; Grzanna and Coyle,

1976), from human pheochromocytoma (Stone et al., 1974), human serum (Miras-Portugal et al., 1975) and bovine brain (Matsui et al., 1982).

Based on sedimentation equilibrium data bovine adrenal DBH has a molecular weight of 290,000 (Friedman and Kaufman, 1965). Sedimentation equilibrium in the presence of guanidine-HCl or guanidine-HCl and diethylthiocarbamate has been used to determine the molecular weight of the enzyme subunits. It has been calculated from these data that the molecular weight is 1.6×10^5 for nonreduced and 7.6×10^4 for reduced enzyme. This suggests the existence of 4 subunits, two which are linked by covalent disulfide bonds and two of which are linked by noncovalent bonds (Craine et al., 1973; Foldes et al., 1973). Electrophoresis on sodium dodecyl sulphate (SDS) polyacrylamide gels gives values in agreement with the previous experiments. A single band is obtained in SDS and urea-containing gels, indicating the presence of single species (monomers) (Craine et al., 1973). This model of DBH is widely accepted (Frigon et al., 1981). Foldes et al. (Foldes et al., 1973) treated enzyme preparations from bovine adrenal medulla with either 8 M urea or 0.1% (w/v) SDS, and obtained dissociation of the enzyme into three similar subunits, each of molecular weight of the order of 100,000.

The subunit structure of a membrane-bound form of DBH from bovine adrenal-medulla chromaffin granules has been investigated by immunochemical and electrophoretic techniques (Helle, 1971 a,b). The soluble and bound enzymes have similar molecular weights, viz. 290,000. The bound enzyme can be solubilized with detergents. Helle claims that the two types of the enzyme contain six subunits held together by electrostatic bonds. Urea or SDS did not have any further dissociating

effect on the three subunits (Foldes et al., 1973).

1.A.4. Forms of DBH

DBH in chromaffin granules of the adrenal medulla occurs 50% in a water-soluble form and 50% in a membrane-bound form (Smith and Kirshner, 1967). These two forms are similar in immunoreactivity (Goldstein et al., 1965; Skotland and Flatmark, 1979), mobility during polyacrylamide gel electrophoresis (Hortnagl et al., 1972) and in their binding affinity for various substrates (Brodde et al., 1976). Some differences have been reported in regard to: (i) Arrhenius kinetics depicting discontinuities in the temperature-activity curves (Aunis et al., 1977); (ii) detergent binding (Bjerrum et al., 1979); (iii) amphiphilicity by charge-shift electrophoresis (Winkler et al., 1970); (iv) altered mobility by SDS gel electrophoresis (Sabban et al., 1983); (v) the presence of extractable phospholipids, especially phosphatidylserine or binding of the enzyme to synthetic lipids (Saxena and Fleming, 1983); and (vi) the presence of extra, hydrophobic peptides in the bound form (Slater et al., 1981). However, there are great similarities in the primary structure of the two forms of the enzyme in human and bovine adrenal as revealed by amino acid analysis and tryptic peptide mapping (Sokoloff et al., 1985). Perhaps, differences in secondary or tertiary structure determine the differences in binding to detergents or membranes. This has been shown for other enzymes, such as lactate dehydrogenase (Kimura and Futai, 1978) and pyruvate oxidase (Schrock and Gennis, 1977).

1.A.5. Kinetics

Tyramine is a better substrate for the enzyme in vitro than is DA or phenylethylamine (Molinoff et al., 1971; van der Schoot and Creveling, 1965). The apparent Km values of the enzyme for tyramine,

ascorbate and fumarate are 2.0×10^{-3} M; 0.9×10^{-3} M; and 0.6×10^{-3} M respectively (Foldes et al., 1973). Km values ranging from 1.7×10^{-4} M to 7.0×10^{-3} M have also been reported for the enzyme with tyramine (Belpaire and Laduron, 1968). The hydroxylation proceeds by a "ping-pong" mechanism (Goldstein et al., 1968; Foldes et al., 1972). Neither AD nor NA has any inhibitory effect on the enzyme activity in vitro at any of the concentrations tested (0.2-8,4 mM). Addition of low concentrations of cupric ions stimulates the catalytic activity up to an optimum concentration around 0.03 mM. Above this, inhibition is observed (Foldes et al., 1973; Goldstein and Contrera, 1968; Molinoff et al., 1971; Coyle and Axelrod, 1972).

1.A.6. Ascorbic acid as cofactor

The best documented specific role for ascorbate is in nervous system tissue where it functions as cofactor for DBH (Friedman and Kaufman, 1965). This enzyme requires an electron donor; ascorbic acid serves in this capacity (Levin et al., 1960). In fact, recent studies have suggested that ascorbate can modulate neurotransmission; that is, ascorbate inhibits dopamine-sensitive adenylate cyclase in vitro (Thomas and Zemp, 1977); blocks amphetamine-induced stereotypic behaviour in rats (Tolbert et al., 1979); and inhibits the binding of neurotransmitters to their receptors (Leslie et al., 1980).

The physiological role of ascorbate in beta-hydroxylation is supported by (i) the presence of high concentration of this vitamin in chromaffin vesicles (Ingebretsen et al., 1980); and (ii) the fact that primary cultures of bovine adrenomedullary cells accumulate ascorbic acid and release it under stimulation by nicotine along with catecholamines (CA) (Daniels et al., 1982). This release is a Ca^{2+}-dependent

process. Thus, chromaffin cells have a mechanism to accumulate ascorbic acid at the site of DA beta-hydroxylation. The vitamin would be taken up from the circulation and could be conserved during physiological function through the putative cytoplasmic regeneration system (Diliberto and Allen, 1980).

1.A.7. Inhibitors

Several substances inhibit DBH. Diethyldithiocarbamate, a well-known copper-chelating agent, is effective *in vitro* and *in vivo* (Goldstein et al., 1964). [bis-(1-Methyl-4-homopiperazinyl-thiocarbonyl)-disulfide] (Fla-63) and methimazole act by the same mechanism (Anden and Fuxe, 1971; Stolk and Hanlon, 1973). Fusaric acid (5-butylpicolinic acid) and several of its analogues are potent inhibitors *in vitro*, apparently acting by means other than copper-binding; they have hypotensive actions (Hidaka et al., 1969, 1973). The inhibition by fusaric acid is uncompetitive with the substrate, but competitive with the ascorbic acid cofactor. Substrate analogues, such as benzyloxyamine and benzylhydrazine, are also potent inhibitors *in vitro* (Hidaka et al., 1973).

Because Cu^{2+} at the active site of the enzyme undergoes cyclic oxidation and reduction (Friedman and Kaufman, 1965) it is thought that the action of at least some inhibitors is exerted by interaction with Cu^{2+} (Nagatsu et al., 1967, Orcutt and Molinoff, 1976). The inhibition of adrenal activity is reversed by N-ethylmaleimide and by Cu^{2+} (Duch et al., 1968).

The observation that little or no DBH activity is present in homogenates of adrenal glands, but does show up in fractions of the homogenate, led to the suggestion that some native substance limits the hydroxylation *in vivo* (Creveling et al., 1962). Indeed, endogenous

inhibitors of DBH exist in many tissues (Nagatsu et al., 1967).

Endogenous inhibitors of this enzyme have been purified from heat-denaturated homogenates of heart (Chubb et al., 1969) and from bovine adrenal medulla (Nagatsu et al., 1967; Duch et al., 1968). It has been thought that the inhibitors could be glutathione and cysteine (Nagatsu et al., 1967; Duch et al., 1968). However, depletion of glutathione with diethylmaleate does not completely eliminate inhibitory activity in homogenates of adrenal gland (Orcutt and Molinoff, 1976). These authors have reported differences in the endogenous inhibitors from several rat organs.

1.A.8. Metabolic clearance

DBH enters the circulatory compartment with the sympathetic neurotransmitters presumably after exocytotic release from nerve cells (Smith et al., 1970; Weinshilboum et al., 1971). Studies of serum DBH activity and its relationship to nervous function in a variety of species show that circulating levels of DBH do not correlate with sympathetic neuronal activity (Kopin et al, 1976). This failure of serum DBH activity to reflect acute changes in sympathetic nervous system activity is well illustrated by studies in insulin-induced hypoglycemia (Nisula and Stolk, 1978) and in subjects with signs and symptoms of altered sympathetic activity followed longitudinally (Friedman and Stolk, 1978). Actually the DBH level in serum reflects the balance between the rate of entry into the serum and the rate of peripheral disposal, so that attempts to correlate DBH activity with sympathoadrenal function without taking in account the disposal pathways will be unsuccessful.

There is also uncertainty about the turnover rate of DBH. The use

of immunochemical and radiolabeling techniques shows that the half-life of DBH in the rat is relatively long (3-5 days). Thus, Grzanna and Coyle (1977) observed that the injection of anti-rat DBH antiserum into rats causes a rapid elimination of DBH activity from the circulation and they calculate the turnover in the serum by the rate of reappearance of circulating DBH. Geyer and Schanberg (1982) obtained similar values with radioiodinated DBH, used to assess the turnover rate of circulating DBH activity in the rat. This method has been criticized as invalid by Stolk. He and his colleagues have employed a pulse-dose of homologous or heterologous DBH, with measurement of its rate of disappearance (Stolk et al., 1980; Hurst et al., 1982). His studies of the kinetics of metabolism of rat and bovine DBH in the rat (Stolk et al., 1983) indicate that the time-frame of turnover of enzymatically active DBH is hours, not days. In other experiments the enzyme has been infused at a constant rate into the rat circulation (Stolk et al., 1983). Metabolic clearance rates with the two procedures - constant infusion and pulse-dose technique - were similar. Injected DBH activity was cleared at the rate of 1 ml/h per 100g body weight for homologous and 8 ml/h per 100 g body weight for heterologous enzyme, with a mean transit time of about 8 hours.

1.A.9. Methods of determining DBH activity

The early assays used to measure DBH activity were time-consuming and relatively insensitive. One of the most common methods was based on the measurement of the beta-hydroxylated product of DA or tyramine (Creveling et al., 1962). NA formed from DA could be measured fluorometically or chromatographically (Levin et al., 1960). Octopamine formed from tyramine could be cleaved with periodate to yield p-hydroxybenzaldehyde; this product would then be measured spectrophotometrically

or radiometrically (Pisano et al., 1960; Friedman and Kaufman, 1965). Because of their low sensitivity these methods are useful only in the adrenal gland (Nagatsu et al., 1968), which is rich in DBH. An improved spectrophotometric assay (Nagatsu and Udenfriend, 1972; Kato et al., 1978) has a sensitivity of 250 pmol. Activity values with this method are Vmax, because it is carried out at saturating concentrations of substrate (20 mM tyramine).

Some of the radiometric assays are the following: (i) Levin and coworkers (Levin et al, 1960) reported a method that uses (1-^{14}C)dopamine as substrate. The product of the reaction is cleaved to yield ^{14}C-formaldehyde and separated by dimedon precipitation. (ii) The method of Pisano and colleagues (Pisano et al., 1960), makes use of (ring-^{3}H)tyramine as the substrate, which product of the reaction is submitted to perioidate cleavage to produce p-^{3}H-hydroxybenzaldehyde, separated by solvent extraction. (iii) In Creveling's assay (Creveling and Daly, 1965), (β,β-^{3}H)tyramine is the substrate. The ^{3}HOH formed is obtained by separation through Dowex-50 resin. (iv) The method of Molinoff (Molinoff et al., 1971) is explained in detail below.

This radioassay, developed by Molinoff (Molinoff et al., 1971) is highly specific and even more sensitive than the others (30 pmol) and, once the conditions are established, the method is rapid and convenient. The assay involves two reactions: 1. Tyramine is beta-hydroxylated by DBH present in the supernatant of adrenal gland homogenate. 2. The octopamine formed is labeled with ^{14}C-SAM to form synephrine, catalyzed by phenylethanolamine N-methyltransferase (PNMT) partially purified from bovine adrenal medulla. The final radioactive product is separated by solvent extraction and its radioactivity is determined. The maximal

activity of DBH in adrenals is increased to some extent as the dilution of the homogenate is increased, probably due to the dilution of endogenous inhibitors (Molinoff et al., 1971). Therefore, cupric ions (Molinoff et al., 1971) or N-ethylmaleimide (Goldstein, 1971) must be used in the first step (DBH reaction) to inactivate the inhibitors. An optimal concentration of Cu^{2+} has to be determined, according to the dilution of the tissue. Higher concentrations of Cu^{2+} are necessary for more concentrated homogenates. EDTA added in the second step chelates the excess of Cu^{2+} and favors the PNMT reaction.

Two factors are responsible for the high sensitivity achieved with the Molinoff method: i) the ability to assay DBH at substrate concentrations above the Km (2 Km), and ii) the use of a partially purified enzyme in the second step. However, tyramine inhibits the PNMT reaction and DBH can not be saturated with the substrate. Therefore, values obtained by this procedure are not maximum velocities.

Recently, high performance liquid chromatography (HPLC) has been introduced in the assay of various bioactive substances, because of its simplicity, specificity and sensitivity (Kissinger et al., 1981).

Methods to assay DBH by HPLC with fluorescence detection when tyramine is the substrate (Flatmark et al., 1978); with ultraviolet spectrophotometric detection when DA is the substrate (Lasala and Coscia, 1979); and with electrochemical detection when DA is substrate (Davis and Kissinger, 1979; Sperk et al., 1980) have been reported. A more sensitive HPLC method has also been reported, in which NA formed from DA is isolated by a double-column procedure. This method has a sensitivity of 30 pmol, which is comparable to the radiometric assay, and has been used for determination of DBH in cerebrospinal fluid (Matsui et al., 1981).

The problem with HPLC methods is that one must use an excess of substrate to ensure zero order kinetics with respect to substrate. Hence when the reaction is stopped a considerable amount of unreacted substrate remains. In the method with DA as substrate the hydroxyl group in the beta position of the side chain has a large effect in reducing the hydrophobicity of NA relative to DA; this is especially important in reversed phase HPLC modified with ion-pair reagents. Resolution of the two compounds is accomplished with a difference of 10 min in retention times. Thus, one is obliged to wait for the DA to be eluted before another sample can be injected. In addition, the large amount of DA (substrate) injected overloads the column and saturates the output of the detector. These last problems can be resolved by utilizing split column chromatography (Davis and Kissinger, 1979). This involves the use of two short columns instead of a single longer one; a valve between the two columns is opened for DA to be wasted.

Despite advantages of the HPLC methods, the radioenzymatic method of Molinoff (Molinoff et al, 1971) offers facile operation, specificity, sensitivity and rapidity, and is ideal for DBH determination. It was selected for the work described in this thesis.

1.B. Phenylethanolamine N-methyltransferase

Phenylethanolamine N-methyltransferase (PNMT, EC 2.1.1.28), the enzyme that catalyzes the conversion of NA to AD (Kirshner and Goodall, 1957; Axelrod, 1962) is highly localized in the adrenal medulla but is also present in the CNS (Ciaranello et al., 1969; Saavedra et al., 1974; Hökfelt et al., 1973; Yu, 1978). This enzyme was first reported by Kirshner and Goodall (1957) to occur in the adrenal medulla and to use SAM as methyl donor. Axelrod (1962) purified the enzyme further. PNMT

acts upon a variety of phenylethylamines, but these require a hydroxyl group in the beta position of the side chain. The molecular weight of PNMT, by Sephadex chromatography, is 38,000; no cofactors are needed (Connett and Kirshner, 1970). Titration of the enzyme with p-hydroxymercuribenzoate showed that there are 8.5 moles of sulfhydryl groups, but only two are essential for catalytic activity (Connet and Kirshner, 1970). Kinetic analysis reveals a complex reaction mechanism with random binding of substrates (Connett and Kirshner, 1970) or preference of SAM as the first substrate bound (Pendleton and Snow, 1973). The method of determination developed by Axelrod (1962) is based on the incubation of the enzyme with normetanephrine and ^{14}C-SAM. The N-methyl-^{14}C-metanephrine formed is extracted into a mixture of toluene and isoamyl alcohol at pH 10 and the radioactivity in the solvent is measured. Octopamine can also be used as substrate (Díaz Borges et al., 1978; Yu, 1978). A HPLC method has been used for determining PNMT with NA as substrate and with electrochemical detection of the product (Borchardt et al., 1979).

1.C. Regulation of catecholamine biosynthesis

CA are synthetized, stored and released from the central nervous system (CNS), sympathetic ganglia, sympathetically innervated organs and adrenal medulla. Under physiological conditions the concentration of tissue CA does not change appreciably. Increased sympathetic activity causes acceleration in synthesis and release of CA in sympathetically innervated structures and adrenal medulla (Bygdeman and von Euler, 1958; Weiner et al., 1973).

Four enzymes are involved in CA biosynthesis: tyrosine hydroxylase (TH), DOPA decarboxylase (DDC), DBH and PNMT (Figure 1). The enzymes

involved in CA metabolism are monoamine oxidase and catecholamine O-methyltransferase (Figure 2) (Cooper et al., 1978).

TH is a soluble enzyme located in the cytoplasm of cell bodies and nerve endings. It is generally considered the rate-limiting step in CA biosynthesis (Levitt et al., 1965). The 3,4-dihydroxyphenylalanine (L-DOPA) produced by TH is rapidly decarboxylated by DDC, or aromatic amino acid decarboxylase, which is also a cytoplasmic enzyme (Sourkes, 1966).

The DA formed is stored in the dopaminergic neurons. In the noradrenergic neurons, DA is taken up by the storage granules and is hydroxylated by DBH (Kirshner, 1957; Livett et al., 1969). In the adrenal gland and in the CNS, NA leaves the storage vesicles to be N-methylated in the cytoplasm by PNMT (Axelrod, 1962; Ciaranello et al, 1969).

The intracellular concentration of NA is of the order of 10^{-4} M (von Euler, 1967), and is thus of the same magnitude as the Ki of TH for NA (Musacchio et al., 1973). Cytoplasmic reduction of DA levels produces an increase in DA synthesis by the release of a feedback inhibition of TH (Glowinski et al., 1973). The administration of pargyline or phenipraxine, two monoamine oxidase inhibitors, produces an elevation of DA levels with a marked reduction of the accumulation of ^{3}H-DA from ^{3}H-tyrosine, and of the activity of TH (Javoy et al., 1972). However, tyrosine administration increases DOPA to a small extent in rat brain, and treatments that decrease brain tyrosine decrease DOPA accumulation (Wurtman et al., 1974). This indicates that TH is about 75% saturated under normal conditions (Carlsson and Lindquist, 1978).

A short-term stimulus, such as a brief increase in neurotransmitter utilization, produces a release of the feedback

inhibition on TH, and a subsequent increase in enzyme activity. After prolonged augmentation of activity in the CA-containing systems there is an increase in the enzyme activities, mediated by an increase in the rate of synthesis (Musacchio et al., 1969; Thoenen, 1970; Reis et al., 1974; Molinoff et al., 1970; Ciaranello and Black, 1971).

Recent data suggest that phosphorylation of TH is a primary cellular mechanism for the control of TH activity. Cyclic AMP-dependent protein kinase (kinase A) phosphorylates and activates the enzyme in vitro (Vulliet et al., 1980). Physiological activation of the adrenergic system produces kinetic changes of TH similar to those seen in vitro (Masserano and Weiner, 1979). Other investigations have indicated that the cofactor for the hydroxylation of tyrosine, L-erythro-tetrahydrobiopterin (Kaufman and Fisher, 1974), is important for the biosynthesis of biogenic monoamines and that monoamines may in turn regulate the biosynthesis of the pterin cofactor from GTP (Nagatsu, 1983). Thus, there is mutual regulation between the biosynthesis of biogenic amines and that of the biopterin cofactor. Drugs that increase NA in vivo inhibit CA synthesis and adding pteridine cofactor to the preparation reverses the effect (Weiner et al., 1973).

Although TH is the rate-limiting enzyme in the biosynthesis of CA, DBH seems to play an important role in the regulation of NA biosynthesis. Disulfiram, a potent inhibitor of DBH in vitro and in vivo (Goldstein, 1966; Goldstein and Nakajima, 1967) causes a rapid and marked fall in endogenous content of NA in brain and heart and elevates endogenous DA. This inhibitor blocks the replenishment of NA stores after depletion by reserpine (Goldstein and Nakajima, 1967). In a time-course study, disulfiram proved more effective than alpha-methyl-p-tyrosine (AMPT), an inhibitor of TH, in decreasing NA in brain

(Goldstein and Nakajima, 1967).

1.D. Short and long term stimulation of CA biosynthesis. Molecular characteristics of tyrosine hydroxylase, dopamine beta-hydroxylase and phenylethanolamine N-methyltransferase

Acute stimulation of the splanchnic nerve increases both the release and the biosynthesis of CA in the adrenal medulla (Bygdeman and von Euler, 1958). Recent data suggest that the phosphorylation of TH is primary in this effect. cAMP-dependent protein kinase (kinase A) phosphorylates and activates the enzyme in vitro (Vulliet et al., 1980) and in vivo (Masserano and Weiner, 1979; Waymire, 1984). Direct stimulation of the splanchnic nerve also activates adrenal TH (Vulliet et al., 1984).

Chronic stimulation of the splanchnic nerve leads to an increase in the synthesis of enzymes of CA biosynthesis, TH, DBH and PNMT; they increase in activity in a coordinated fashion (Ross et al., 1978, 1979). This suggests that similar mechanisms control the regulation of the genes coding for these enzymes. On the other hand, the three enzymes are not expressed in all CA neurons; this would mean that separate genes code for each enzyme and that the separate genes are independently expressed. Aminoacid analysis and peptide mapping of the three enzymes show that they share common protein domains in their primary structures (Joh et al., 1981). Immunochemical and structural analysis of CA-synthesizing enzymes implies that they may also share common gene-coding sequences. cDNA cloned for each enzyme cross-hybridizes with mRNA of the three enzymes (Joh et al., 1984). Joh has proposed that the homologies of these CA enzymes gene-coding regions may be the result of duplication of a common ancestral gene, followed by divergent evolution,

though with conservation of sequences essential for catalytic activity (Joh, 1985).

1.E. Transsynaptic induction of adrenal enzymes. Molecular mechanism

In their studies of the transsynaptic induction of TH Costa and Guidotti (Guidotti and Costa, 1973; Costa, 1976) showed that the activation of nicotinic receptors produces an increase in cAMP and protein kinases. These protein kinases are taken up into the nucleus where they promote the production of mRNA; this increases the rate of synthesis of specific proteins (Joh, 1973). Reserpine administration produces the transneuronal induction of adrenal TH (Thoenen, 1970) and increases cAMP (Guidotti and Costa, 1976), protein kinase activity (Insel, 1975; Costa and Guidotti, 1978) and TH-mRNA (Mallet et al., 1983). Immobilization stress (Paul et al., 1971) and cold exposure (Guidotti et al., 1976) also elevate cAMP levels in the adrenal gland of the rat. Denervation of the gland blocks the increases of TH activity, cAMP and protein kinase activation (Guidotti and Costa, 1973; Insel, 1975; Guidotti et al., 1976). In chromaffin cell cultures cAMP induces TH (Kamamura et al., 1979). It has been proposed that phosphorylation of acidic proteins could inhibit the chromatic template restriction and increase the synthesis of specific RNA (Costa and Guidotti, 1978).

1.F. Release of CA from the adrenal medulla

The adrenal medulla is composed largely of chromaffin cells. These cells have no terminals, but receive innervation from spinal cord segments T_9-T_{11} through the adrenal branch of the splanchnic nerves (Hillarp, 1947). ACh is the physiological mediator of excitation of the

adrenal medulla (Feldberg et al., 1934; Trifaro et al., 1972; Costa et al., 1981). Nicotinic and muscarinic receptors are present (Kirshner and Viveros, 1972). ACh causes depolarization of the plasma membrane in the absence of Ca^{2+}, but secretion occurs only in the presence of that ion (Douglas, 1968). External Na^{+} is necessary also, probably to effect changes in membrane potential (Banks et al., 1979). Finally, a metabolic source of energy is necessary (Kirshner and Smith, 1969). Release of CA from the adrenal medulla occurs by exocytosis, a process in which the content of the adrenal storage vesicles is released to the exterior, leaving the membrane of the vesicle within the cell (Poisner and Trifaró, 1967; Viveros et al., 1969; Baldessarini, 1975). The energy source for uptake and sequestration of CA and nucleotides is a proton-pumping ATPase providing a pH gradient (low inside the vesicle) and a membrane potential. Except for the complexes between ATP and CA, the granule content is iso-osmotic with the cytoplasm (Kirshner and Viveros, 1972). Studies with primary cultures of adrenal medulla have facilitated the understanding of some of these phenomena (Kirshner and Viveros, 1972; Trifaró and Bouner, 1981). The cultured chromaffin cells have a secretory behaviour like the cells in the intact gland (Trifaró and Lee, 1980).

It has been hypothesized that in the splanchnic nerve ACh coexists with opioid peptides and that these substances are coreleased, each acting on its respective receptors in the chromaffin cells (Schultzberg et al., 1978). It seems that these peptides then modulate the release of CA elicited by the action of ACh (Costa et al., 1980), by inhibiting it. This inhibition is non-competitive, and probably involves an indirect mechanism (Kamakura et al., 1980).

Chromaffin cells also contain GABA and benzodiazepine receptors;

these are functionally linked (Costa et al., 1984). The action of muscimol on CA release is blocked by bicuculline; this suggests that $GABA_A$ receptors occur. GABA is said to have an inhibitory action on CA release (Costa et al., 1984).

1.G. Adrenal cortex and medulla. Regulation of adrenal enzymes

The adrenal cortex is of mesodermal origin and produces steroids. Corticotropin releasing factor (CRF) from the hypothalamus acts on the anterior pituitary and stimulates the production and release of adrenocorticotropin (ACTH) which, through the circulation, gets to the cortex and stimulates the synthesis and release of glucocorticoids. The adrenal medulla derives from the neural crest (ectoderm) and produces CA. There is some evidence for splanchnic innervation to the adrenal cortex (Unsicker, 1971) and for a neural role in the secretion of corticoids (Henry et al., 1976). The adrenal cortex and medulla are intimately related not only anatomically, but functionally. For example, splanchnic innervation of the medulla is not the only regulator of its functions; adrenal cortical hormones and ACTH play regulatory roles in the production of medullary CA (Ciaranello, 1980).

In response to stressful stimuli the adrenal gland secretes CA into the circulation from the medulla, and glucocorticoids from the cortex. Both hormones increase the available body glucose by promoting glycogenolysis and gluconeogenesis, respectively (Figure 3). Also, NA and AD have vasoconstrictive properties, and glucocorticoids stabilize epithelial membranes.

1.G.1. Humoral control

The levels of PNMT in the adrenal medulla are reduced in hypophysectomized rats and restored by hydrocortisone or dexamethasone

(Coupland, 1953; Wurtman and Axelrod, 1966). Also glucocorticoids seem to be important in regulating the resting activity of other enzymes, such as TH (Mueller et al., 1970), DBH (Weinshilboum and Axelrod, 1970), catechol-O-methyltransferase and monoamine oxidase (Wurtman and Axelrod, 1966). Only minor changes in ultrastructure of the adrenal medulla are observed after hypophysectomy (Pohorecky and Rust, 1968).

1.G.2. Neural control

Reflex activation of the splanchnic nerves to the adrenal medulla by agents such as reserpine or 6-hydroxydopamine (6OHDA) elevates adrenal TH (Mueller et al, 1970), DBH (Molinoff et al., 1970) and PNMT (Ciaranello and Black, 1971). Other treatments such as administration of dopaminergic agonists transsynaptically increase adrenal TH (Quik and Sourkes, 1976) and adrenal ornithine decarboxylase (Almazan et al., 1980). Immobilization and cold stress also induce these adrenal enzymes (Thoenen, 1971; Kvetňansky et al., 1970, 1971). Direct stimulation of the nicotinic receptors on the chromaffin cells with ACh (Patrick and Kirshner, 1971) or carbamylcholine (Guidotti and Costa, 1973) results in an increase of these enzymes in the rat.

1.G.3. Dual regulation of dopamine beta-hydroxylase and phenylethanolamine N-methyltransferase

Studies of DBH (Ciaranello et al., 1975) and PNMT (Ciaranello et al., 1978) by immunotitration and by double labeling of the enzymes with pulses of ^{3}H and ^{14}C-aminoacids show that these enzymes are regulated in a dual manner. Both neuronal and humoral stimuli affect them, but by different biochemical mechanisms: transsynaptic factors control enzyme induction (synthesis) and hormonal control regulates steady state levels

(degradation). In contrast to the action of glucocorticoids or ACTH, which only restores the enzymes back to control levels in hypophysectomized rats (Weinshilboum and Axelrod, 1970), the neuronal stimulation increases them in normal animals. Moreover, it has been shown that ascorbic acid and SAM, cofactors of DBH and PNMT reactions respectively, prevent proteolytic breakdown of these enzymes (Wong and Ciaranello, 1981, 1982). Both cofactors decrease after hypophysectomy in the adrenal gland (Wong and Ciaranello, 1981, 1982).

1.H. Coexistence and interaction of neurotransmitters

Monoamines are localized in the CNS in cell groups termed A_1-A_{12} for CA and B_1-B_9 for serotonin (5HT) (Dahlström and Fuxe, 1964). These neurons represent only a very small proportion of the cells in the CNS (Dahlström and Fuxe, 1964). In areas with a very high concentration of CA fibers only 10-15% of all nerve endings contain the monoamine (Hökfelt, 1968). Thus, other neurotransmitters could coexist in the same neuron or area of the brain and interact with each other. On the basis of dynamic histoimmunochemistry in central CA and peptide systems Hökfelt (1984) assumes that those interactions could be: 1. Direct: CA and peptides contact with each other at different levels. 2. Indirect: This could occur by separated axo-somatic contact in a third neuron or by intermediation of an interneuron. 3. Coexistence: Monoamines and peptides coexist in some neurons of the brain. Simultaneous release of both neurotransmitters and neuromodulators could affect the postsynaptic response or the presynaptic regulation, as has been reported in peripheral models (Lundberg et al., 1982).

In addition, the fact that some central functions are modulated by several neurotransmitters acting in a facilitatory or inhibitory way

suggests that they interact to modulate those functions in a balanced manner. Some examples of these interactions are:

1) Adrenergic-cholinergic interaction: Evidence of this type of interaction is as follows: (i) a noradrenergic inhibitory system activated by ACh has been reported to be present in the cerebral cortex of rats (Malcolm et al., 1967); (ii) ACh and 5HT are shown to have positive effects on DA release from caudate nucleus (Glowinski, 1970); (iii) depletion of striatal ACh produced by reserpine is reversed, by administration of L-DOPA (Beanie et al., 1966).

2) Serotonergic-dopaminergic interaction: This interaction is suggested in various reports: (i) psychotic manifestations produced by 5HT are reported to be decreased by L-tryptophan and by L-DOPA (Birkmayer et al., 1972); (ii) administration of L-DOPA produces an increase in DA, but also a marked decrease in brain 5HT (Everett and Borcherding, 1970; Ng et al., 1970); (iii) there is a central serotonergic-dopaminergic interaction in the regulation of adrenal TH activity (Quik and Sourkes, 1977), in which the DA system is stimulatory and 5HT is inhibitory.

3) Cholinergic-serotonergic interaction: The following evidence supports this interaction: (i) 5HT in vitro inhibits brain AChesterase in a competitive manner (Mohammed et al., 1975); (ii) some cholinergic agonists increase 5-hydroxyindolacetic acid (5HIAA), a 5HT metabolite in the brain (Haubrich and Reid, 1972).

4) Gabaergic-dopaminergic interaction: Iontophoretic application of gamma-aminobutyric acid (GABA) to neurons in the substantia nigra produces neuronal inhibition of ACh and DA neurons; this is blocked by picrotoxin (Groves et al., 1973).

5) Gabaergic-noradrenergic-serotonergic interaction: It has been reported that GABA treatment lowers NA and raises 5HT levels in the brain; this is more pronounced in the hypothalamus (Yessain et al., 1969).

6) Gabaergic-cholinergic-dopaminergic interaction: Picrotoxin, a $GABA_A$ receptor blocker, produces an increase in ACh in rat striatum, which is antagonized by AMPT. It has been suggested that by blocking GABA receptors DA activity is increased and inhibits cholinergic neurons leading an increase in ACh content (Ladinsky et al, 1976).

7) Catecholaminergic-catecholaminergic interaction: Antelman and Caggiula (1977) have described a NA system that when is depressed brings about a facilitation of DA function.

Other examples specifically related to the present work are given in the Results and Discussion section.

1.I. Pathways of stress

Walter Cannon in 1911 introduced the concept of homeostasis and describedthe complex physiological reactions that maintain the internal equilibrium of the organisms. Cannon demonstrated that, during stress, AD is released from the adrenal glands (Cannon, 1929). Then, it was recognized that the repeated exposure to certain stimuli results in adaptative changes in order to preserve the internal conditions. Selye introduced the concept of the "general adaptation syndrome " with special attention to the role of adrenal glucocorticoids (Selye 1936, 1976). Since then, the hypothalamo-hypophyseal-adrenocortical axis has taken importance in studies of the endocrine response to stress. The stress theory of Selye described a non-specific response, but later research has demonstrated the existence of different patterns of

endocrine response to stress agents, so that there is no single general response to all kinds of stimuli (Mason, 1971).

Despite the fact that changes in the adrenal medulla were well recognized, the role of CA in stress was temporarily forgotten. When more information concerning the central action of CA as neurotransmitters was available, it was inevitable that the action of CA in stress research recovered importance. Some laboratories have been interested in the regulation of certain medullary functions, such as DBH and PNMT activities (Ciaranello 1975, 1982; Wong et al., 1983). Others have worked on the molecular aspects of the adrenal medullary activation (Costa and Guidotti, 1978). And others have studied the nervous pathways regulating these functions and producing a final response through the activation of the adrenal gland (Sourkes, 1983, 1985).

Different types of stimuli have been used, such as immobilization stress (Kvetňansky et al., 1971), cold exposure (Thoenen, 1970) or high temperature conditions (Simmonds, 1969), hypoglycemic shock (Viveros et al., 1969; Patrick and Kirshner, 1971), hemorrhage (Cubeddu et al., 1978) or the action of certain pharmacological agents of known specific action (Thoenen, 1971; Molinoff et al., 1970; Lewander et al., 1977; Sourkes, 1983).

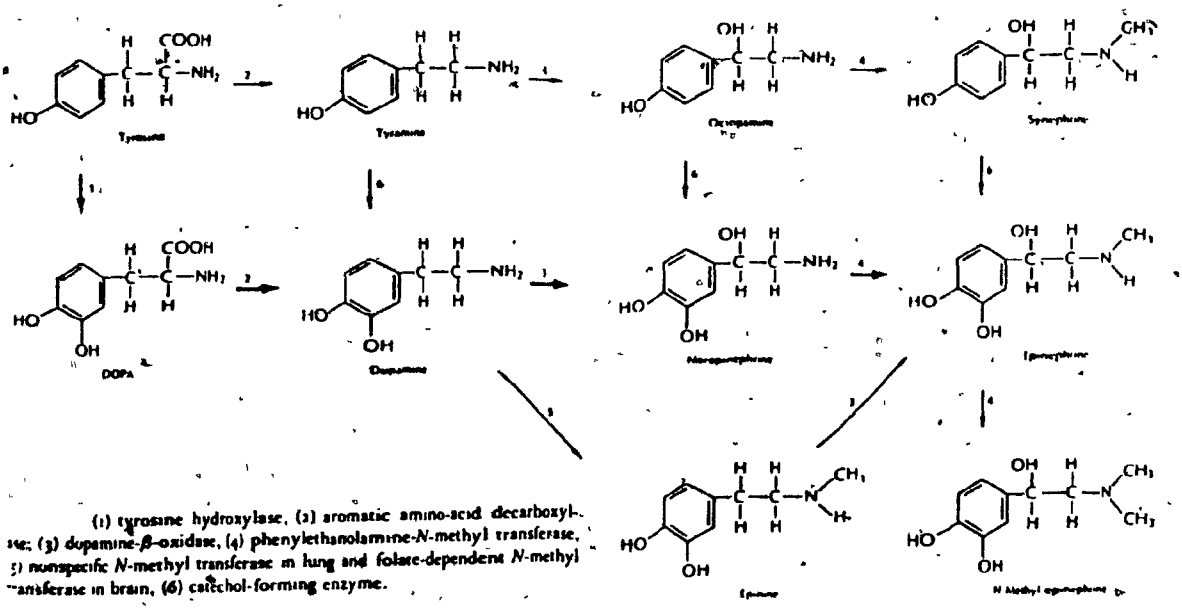
Several central pathways can be activated under pharmacological stress. The administration of DA agonists (Quik and Sourkes, 1976, 1977; Gagner et al., 1983), ACh agonists (Lewander et al., 1977), of reserpine (Thoenen, 1971), or antiserotonergic drugs (Quik and Sourkes, 1977) increases adrenal TH activity. Some of these treatments, such as administration of reserpine (Molinoff et al., 1970) and cholinergic agonists (Lewander et al., 1977), also increase adrenal DBH activity.

The aim of this work is to shed light on the interaction of neurotransmitters related to the dual-regulated adrenal enzyme, DBH.

1.J. Central administration of specific compounds

Intracranial chemical administration of drugs is a valuable tool to extend and refine the method of electrochemical stimulation. The advantages of using the icv route for drug administration are: i) avoidance of the blood brain barrier, in the case of impermeability of certain substances; ii) reduction of collateral effects in the periphery; and iii) restriction of the drug action to specific brain areas. However, some precautions concerning the diffusion of the substance to be administered should be taken (Girgis, 1983; Lum et al., 1984). These are: i) use of an appropriate concentration of the drug to be injected; ii) control of duration of the injection to avoid asymmetric uptake; and iii) administration of the drug in very small volumes to circumscribe its action.

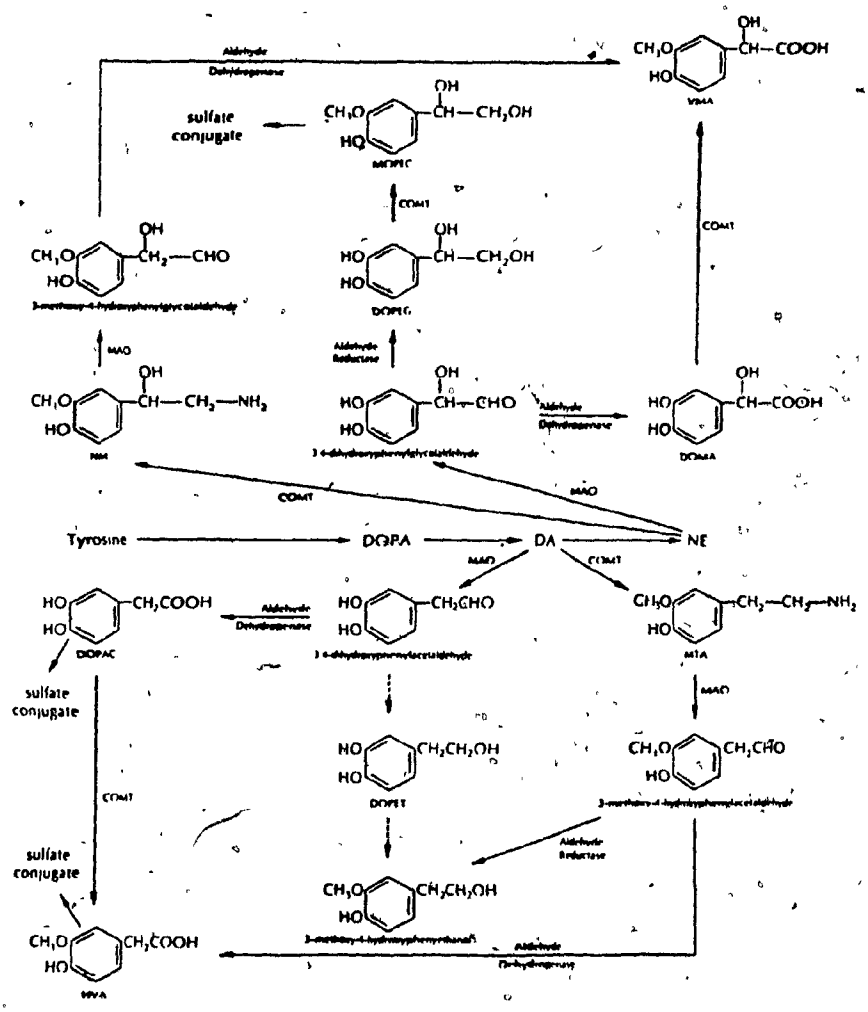
Microperfusion with osmotic minipumps permits the continuous administration of the compound under study (Theevwes and Yum, 1976; Lum et al., 1984). This technique offers the following advantages: i) constant and prolonged administration; ii) less stress of the animal, owing to less handling, and iii) minimum damage of the SNC, because the injection of serial pulses is avoided. In addition, much lower doses are sufficient to obtain a pharmacological effect, in contrast to the acute injections of the tested substance.



(1) tyrosine hydroxylase, (2) aromatic amino-acid decarboxyl-ase; (3) dopamine-β-oxidase, (4) phenylethanolamine-*N*-methyl transferase, (5) nonspecific *N*-methyl transferase in lung and folate-dependent *N*-methyl transferase in brain, (6) catechol-forming enzyme.

Figure 1

Primary and alternative pathways
in the formation of catecholamines.



The following abbreviations are used: DOPA, dihydroxyphenylalanine; DA, dopamine, NE, norepinephrine; DOMA, 3,4-dihydroxymandelic acid; DOPAC, 3,4-dihydroxyphenylacetic acid, DOPEG, 3,4-dihydroxyphenylglycol, DOPET, 3,4-dihydroxyphenylethanol; MOPET, 3-methoxy-4-hydroxy-

Figure 2

Metabolism of dopamine and noradrenaline.

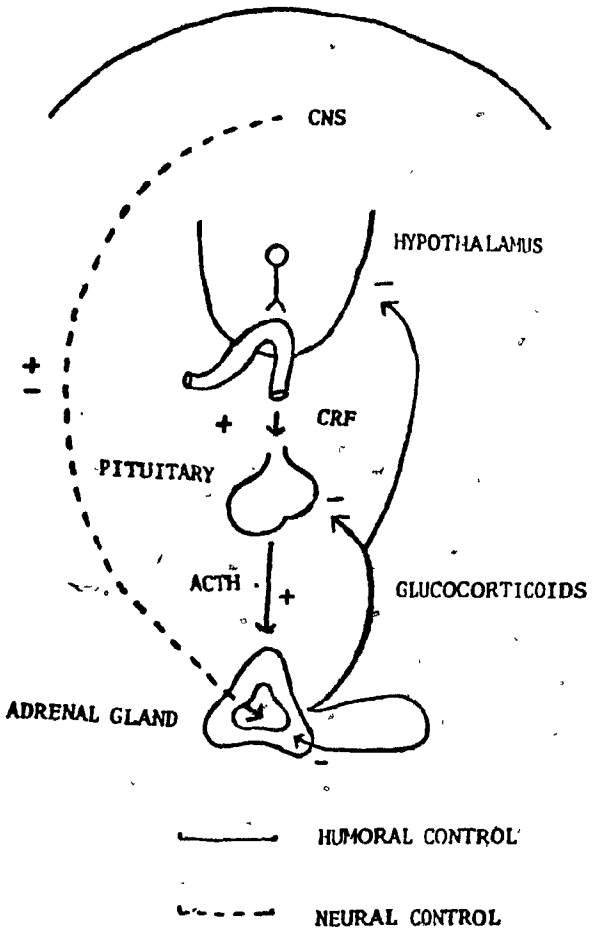


Figure 3

Schematic representation of the humoral and neural pathways to the adrenal gland.

2.- MATERIALS AND METHODS

Information concerning animals used in the experiments and their treatments, surgical and histological procedures, and PNMT purification are specified in corresponding Results and Discussion section. Other methods are described in this section.

2.C. Tissue

2.C.1. Adrenal gland

Adrenal glands were removed from animals deeply anesthetized with sodium methohexital, 65 mg/kg ip, and placed on ice. They were dissected, extracted from the capsule, and weighed. Tissue to be analyzed for enzyme assay or CA determination was homogenized in 1 ml of the appropriate solution by means of 10 passages with a Teflon homogenizer.

2.C.2. Brain

The brain was removed from the skull of animals decapitated after sodium methohexital anesthesia. It was cooled on ice, cleaned and weighed. Homogenization was done by 15 passages of a Teflon homogenizer in four volumes of 0.36 N perchloric acid, containing 0.1% ascorbic acid. The supernatant fraction was used for CA or 5HT determination.

2.C.3. Forebrain and raphe area

Dissection of forebrain and raphe area was done by the procedure of Aghajanian et al. (Aghajanian et al., 1973). The forebrain was obtained through a section from the anterior border of the superior colliculi to the posterior border of the hypothalamus. Cerebellum was separated from the rest of the brain, after which the posterior midbrain was dissected from the remaining tissue by a vertical section through the superior colliculi and the pons. Finally, a block of tissue containing the raphe perikarya was obtained by: 1) horizontal sections through the cerebral aqueduct and through the lower midbrain, below the median raphe nucleus,

and 2) sagittal sections on either side of the lateral borders of the central gray area alongside the dorsal and medial raphe nuclei (Figure 1).

2.F. Enzyme determinations in the adrenal

2.F.1. Dopamine beta hydroxylase

DBH was determined by the two-step assay of Molinoff et al. (1971) (Figure 2) with slight modifications. Supernatant, 200 µl, was added to 120 µl of the incubation medium in a 15-ml stoppered centrifuge tube. The final concentration of the reactants in the incubation medium were: ascorbic acid (pH 6), 4mM; disodium fumarate (pH 6), 40 mM; pargyline, 0.4 mM; tyramine, 1 mM; Cu_2SO_4, 20 µM, concentration sufficient to block endogenous inhibitors for the given concentration of the enzyme (i.e. dilution of tissue); Tris buffer (pH 6), 0.03 M; and 1500 units of catalase. The first step of the Molinoff assay, the DBH reaction, was carried out at 37° C for 20 min; at the end of that the second step, the PNMT reaction, was initiated by the addition of the following substances in a volume of 100 µl: 20 µl of a preparation of PNMT, partially purified from bovine adrenal medulla; EDTA, 13.5 mM; Tris buffer (pH 8.6), 1 M; and 1.7 nmol of ^{14}C-SAM (58.2 mCi/µmol). After 30 min of incubation the reaction was stopped by the addition of 500 ul of 0.5 M borate buffer pH 10 and by rapidly cooling the tubes on ice. The product, ^{14}C-synephrine, was extracted by the addition of 5 ml of toluene:isoamylalcohol (3:2, v:v) and vortexing for 15 sec. After centrifugation of the mixture at 2000 rpm for 10 min, 2 ml of the organic phase were transferred to scintillation vials and evaporated at 70°C. One ml of ethanol was then added to dissolve the residue, and was followed by 10 ml of Liquifluor for radioactive determination. Blanks were prepared with substrate omitted, with boiled enzyme, or with

3 mM fusaric acid added; these did not vary significantly. Each sample was prepared in duplicate, variation between them being 1-5%. An internal standard of 50 ng of octopamine was included in all experiments. Non-dialyzed preparation of DBH was incubated with various concentrations of tyramine, from 0.05 µM - 2 mM; the Km for tyramine was 0.39 mM. The reaction showed linearity with the concentration of the enzyme up to 2.5% (w:v) dilution of adrenal tissue. The dilution used in the assays was 2%. Maximal formation of product was obtained with 20-30 µl of the PNMT extract from bovine adrenal medulla.

Most of the DBH determinations were done with this procedure, however, at the end of this work, the assay was further modified by reducing the volumes to one-tenth of those specified above, except for the partially purified PNMT solution that was reduced to one-fifth. Final concentrations of the reactants were mantained.

2.F.2. Phenylethanolamine N-methyltransferase

PNMT was determined by the method of Yu, (Yu, 1978) with some modifications. Adrenal glands were homogenized with a Teflon homogenizer in 1 ml of an ice-cold solution of 0.05 M phosphate buffer, pH 7.4, containing 0.15 M HCl, 0.1 mM dithiothreitol, and 1 mM EDTA. Homogenates were centrifuged at 10,000 g for 10 min. Supernatant, 20 µl, was added to 30 µl of the incubation medium. The final concentrations of the reactants in this medium were: Tris buffer (pH 8.6), 0.1 M; dithiothreitol, 3 µM; octopamine, 2 mM; and ^{14}C-SAM, 0.8 nmol (58.2 mCi/mmol). The reaction was carried out at 37°C for 30 min, and was followed by the addition of 100 µl of borate (pH 10), 0.5 M; the microtubes containing the mixtures were placed on ice. The methylated product, ^{14}C-synephrine, was extracted by the addition of 500 ul of toluene:isoamyl alcohol (3:2, v:v) and vortexing for 15 sec.

After centrifugation at 2000 rpm for 10 min, 200 µl of the organic phase were transfered to scintillation vials and evaporated at 70°C. One ml of ethanol was added to dissolve the residue, followed by 10 ml of Liquifluor for radioactive counting. The efficiency of the counting was 85-90%. Blanks were prepared by omitting substrate or by inclusion of boiled enzyme; these showed no significant differences. Each sample was prepared in duplicate; variations were less than 2%. Corresponding internal standards of the ^{14}C-SAM were counted in order to calculate product formation. The Km for the substrate determined with concentrations of octopamine from 0.03 mM - 3 mM was 0.38 mM.

2.F.3. Dopa decarboxylase

Adrenal dopa decarboxylase was determined by the method of Awapara and coworkers (Awapara et al., 1964). Carboxyl-labeled substrate yields $^{14}CO_2$ which is trapped as it is produced, on a filter paper impregnated with 100 µl of a mixture of ethyleneglycol monomethyl ether and monoethanolamine (2:1), and placed in a polypropylene well suspended with a rubber stopper in a 25 ml conical flask. The incubation medium consisted of 0.2 ml of 0.077 M phosphate buffer, pH 6.8, 0.2 ml of 0.08 M pyridoxal phosphate, and 0.1 ml of supernatant of adrenal gland, previously homogenized in the same buffer solution. A preincubation of reagents was carried out for 10 min and the reaction was initiated by the addition of ^{14}C-DOPA, 1 mM (51.8 mCi/mmol). Blanks were prepared with boiled enzyme. The reaction was stopped after 5 min of incubation by the addition of 0.5 ml of 5 N H_2SO_4. The filter paper in the center well was then placed in 10 ml of Liquifluor for scintillation counting.

2.F.4. Lactate dehydrogenase

Adrenal lactate dehydrogenase was determined by a spectrophotometric method (Gilford Spectrophotometer 250). Adrenal glands were homogenized in 1 ml of 0.2 M Tris-HCl buffer, pH 7.3. The reaction velocity was calculated by the decrease in absorbance at 340 nm resulting from the oxidation of NADH. One unit of enzyme activity is represented by: ΔA_{340}/min per mg of protein in the reaction mixture x 100. The incubation medium consisted of 2.8 ml of Tris-HCl, buffer pH 7.3; 0.1 ml of 6.6 mM NADH; and 30 mM sodium pyruvate. This mixture was preincubated at 25°C for 5 min and the reaction was started by the addition of 10 ul of the supernatant of adrenal gland. The decrease in absorbance was followed to 60 min after the addition of the enzyme. Proteins were determined by the method of Lowry (Lowry et al., 1951).

2.G. Monoamine determination

CA and 5HT were determined by HPLC according to the procedure of Felice (Felice et al., 1978), and Anderson (Anderson et al., 1981), respectively. The liquid chromatograph and detection system employed was: Waters M-45 pump (Waters Scientific), a Waters U6K injector, and a uBondapak C_{18} reversed phase column (300 mm x 3 mm I.D., 10 um average particle size) (Waters Association, Milford, MA, U.S.A.). The amperometric detection system comprised a Bioanalytical Systems electrochemical controller (LC-4B), a silicone carbon working electrode, a Ag/AgCl reference electrode and a Teflon thin-layer detector cell and reference electrode compartment (Bioanalytical Systems, West Lafayette, IN, U.S.A.). The potential of the working electrode was + 0.7 V for serotonin and +0.6 V for CA, respectively, versus the reference electrode. Background currents of 1-2 nA were observed. Standards were purchased from Sigma (St. Louis, MO, U.S.A.). Stock solutions were made up in double-distilled water, containing 0.1% ascorbic acid. Diluted

standards were prepared daily.

Whole rat brain, forebrain or raphe area was homogenized in 0.36 N $HClO_4$, containing 0.1% ascorbic acid, to yield a final tissue concentration of 25% (w/v). Adrenal glands were homogenized in 1 ml of the same solution. Then they were centrifuged at 15,000 rpm for 30 min. Some of the homogenates received portions of each standard. The recovery of these standards was calculated by subtracting the endogenous content from the loaded sample and comparing this difference with a standard prepared the day of the homogenization and stored under the same conditions at -70° C. The recovery was 90-95%.

Samples for CA determination were submitted to alumina extraction in order to purify the monoamines to be determined. Supernatants obtained after tissue homogenization (500 ul) were added to 100 mg of alumina in 15 ml glass conical tubes containing 1 ml of Tris buffer, 0.5 M pH 8.6. Each sample was vortexed for 15 sec, allow to stand for 5 min, after which the supernatant was aspirated. The alumina was washed with 1 ml of double-distilled water three times, and 1 ml of perchloric acid, 0.2 M, was added. Tubes were vortexed for 20 s, and allowed to stand for 20 min. The supernatant was filtered through a Swinnex filter, 1.3 mm diameter (Millipore). Recovery was 60-65%.

2.G.1. Catecholamines

The mobile phase used in the determination of CA was 95% 0.1 M phosphate buffer, pH 5, containing 0.2 mM sodium octyl sulphate as ion-pair, and 5% methanol. Volumes of 10-50 ul and standards of 0.5-5 ng were injected (Figures 3 and 4).

2.G.2. Serotonin

The mobile phase used in the determination of 5HT was 92% 0.01

sodium acetate buffer, pH 4.25, adjusted with glacial acetic acid, containing 8% methanol. Volumes of 10-50 ul and standards of 0.5-5 ng were injected (Figures 5 and 6).

2.F. Plasma corticosterone determination

Blood was obtained during the course of the experiment by tail bleeding, and at the end of it by heart puncture. Plasma corticosterone was determined by a competitive protein-binding radioassay (Murphy et al., 1963; Murphy and Wagner, 1972). In this method a known quantity of labeled ligand is added to the unknown ligand and the mixture is exposed to a protein that binds the ligand specifically. The amount of labeled free ligand depends on how much ligand, present in standard curves and samples, competes with it for the protein. Internal standards of corticosterone (Sigma Chemical, St. Louis, MO, U.S.A.) of 0.5-16 ng in ethanol were prepared in duplicates. In performing the assay rat plasma, 10 ul, was pipetted in duplicate on a square of filter paper; the paper was placed in a culture tube and 2.5 ml of methylene chloride (Fisher Scientific, Montreal, Quebec) was added. Samples were shaken for 30 min for extraction of corticosteroids. All samples and standards were evaporated on a hot-water bath. Then 1 ml of the binding-protein solution was added to each tube. This solution was prepared as follows: 150 ul of ^{3}H-corticosterone, 50 uCi/ml (New England Nuclear, 85.8 Ci/mmol); 100 ml gel water (0.05%); and 0.25 ml monkey plasma. The rack was incubated at 45°C for 5 min and at 4°C for 1 h. A tube for total counts was also incubated. Still in the cold incubation, 60 mg of Florasil (Fisher Scientific, Montreal, Quebec) was added to each tube, shaken vigorously for 1 min, and centrifuged. Then, 0.5 ml of the supernatant was pipetted into 2 ml of Econofluor for scintillation counting. A representative curve of standards is shown in Figure 7.

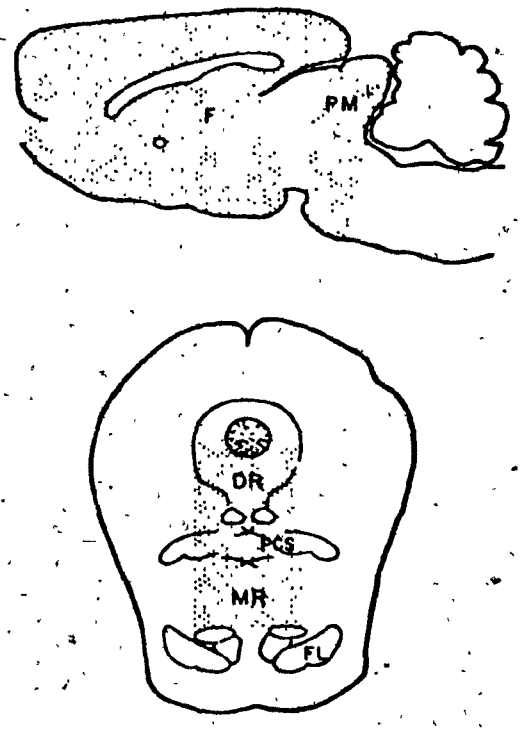


Figure 1

Dissection of raphe area and forebrain

F, whole forebrain; PM, posterior midbrain; DR, dorsal raphe area; MR, medial raphe area; PCS, superior cerebral peduncle.

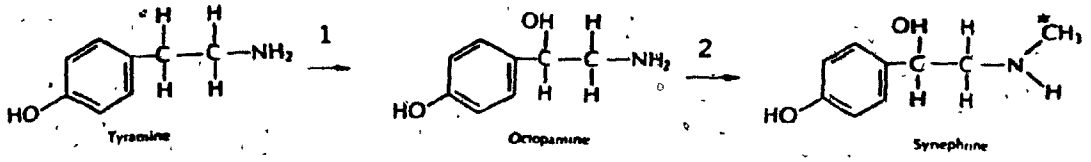


Figure 2

Steps in dopamine beta-hydroxylase assay.

1. First step. DBH reaction.
   Supernatant of adrenal gland homogenate.

2. Scond step. PNMT reaction.
   Enzyme partially purified from bovine adrenal medulla.
   ^{14}C-SAM is the methyl donor.

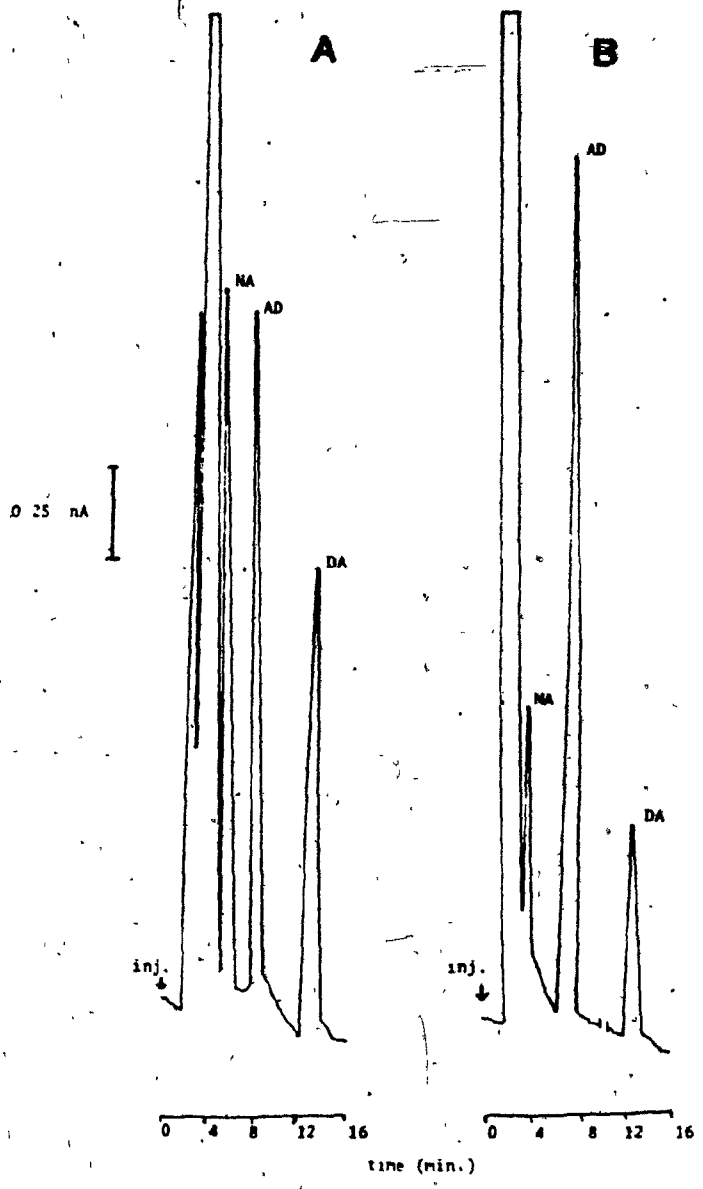


Figure 3

Chromatograms of:
(A) standards of catecholamines
(B) adrenal gland sample.

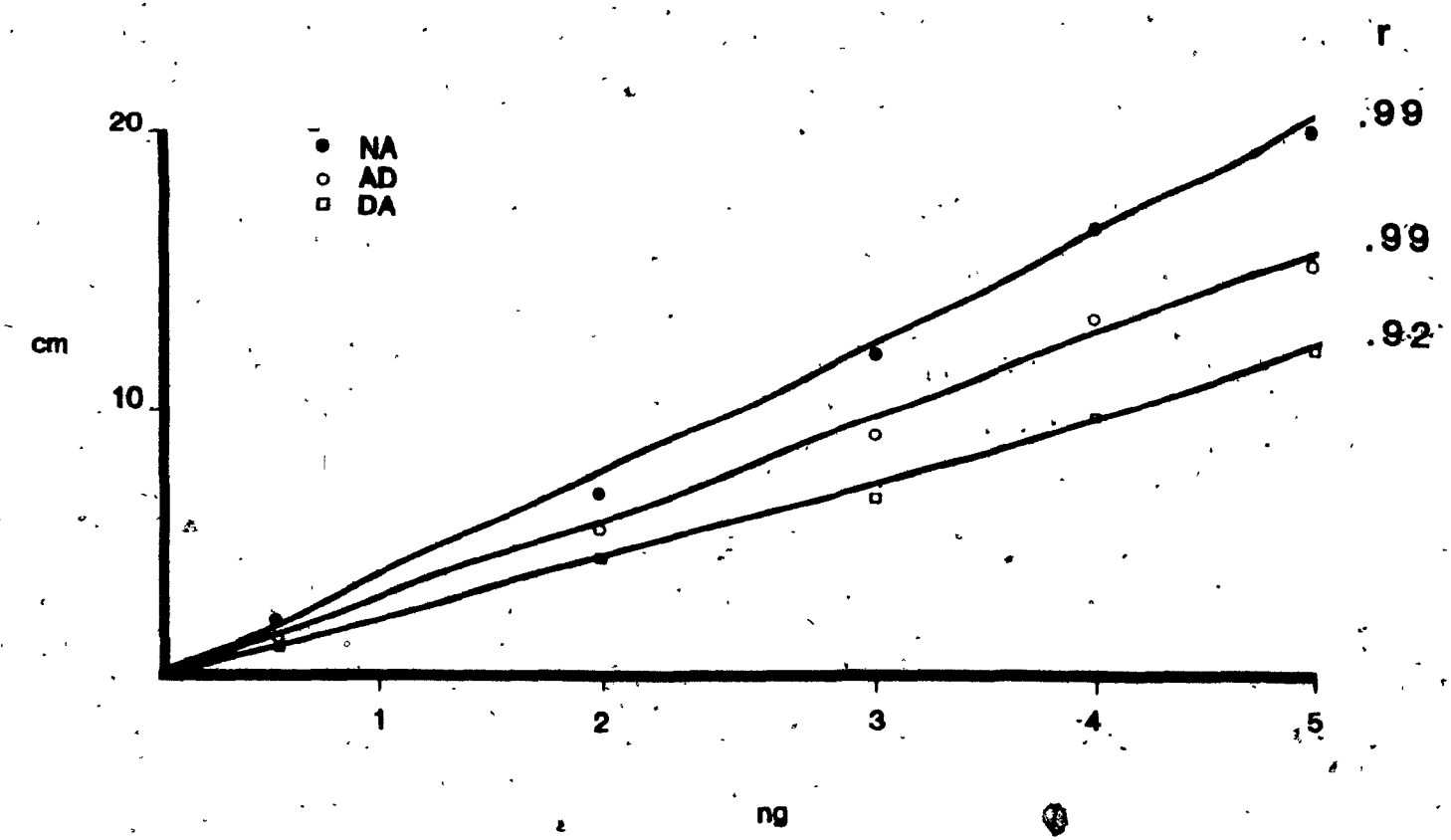


Figure 4

Linear correlation of standards of catecholamines.

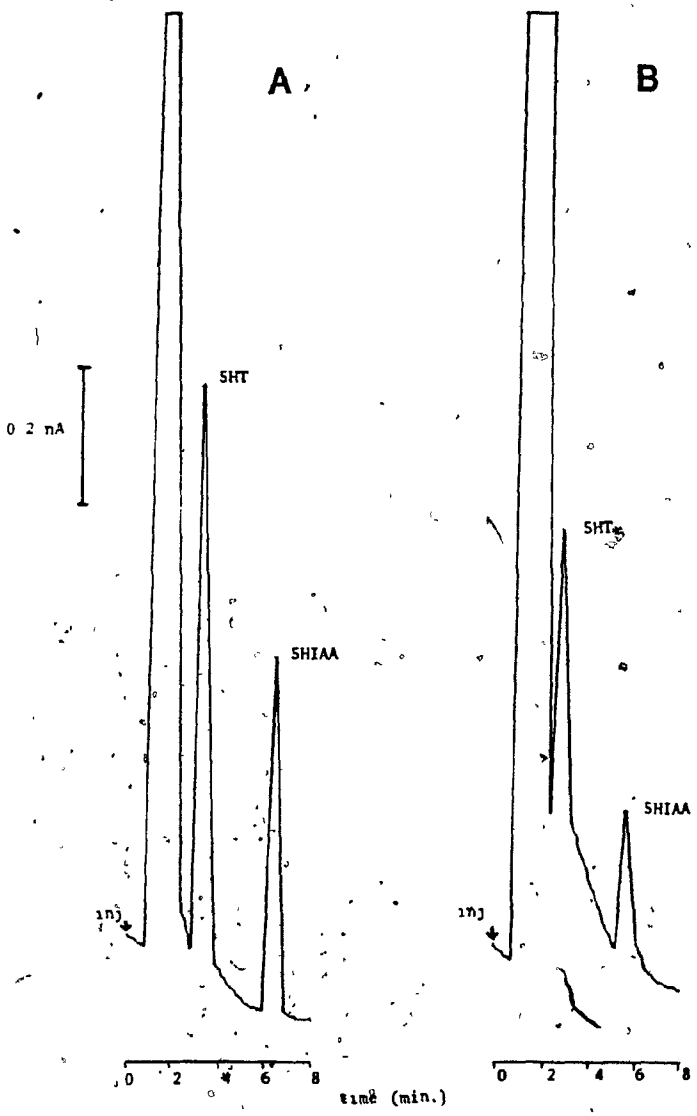


Figure 5

Chromatograms of:

(A) standards of serotonin and 5-hydroxyindolacetic acid

(B) brain sample

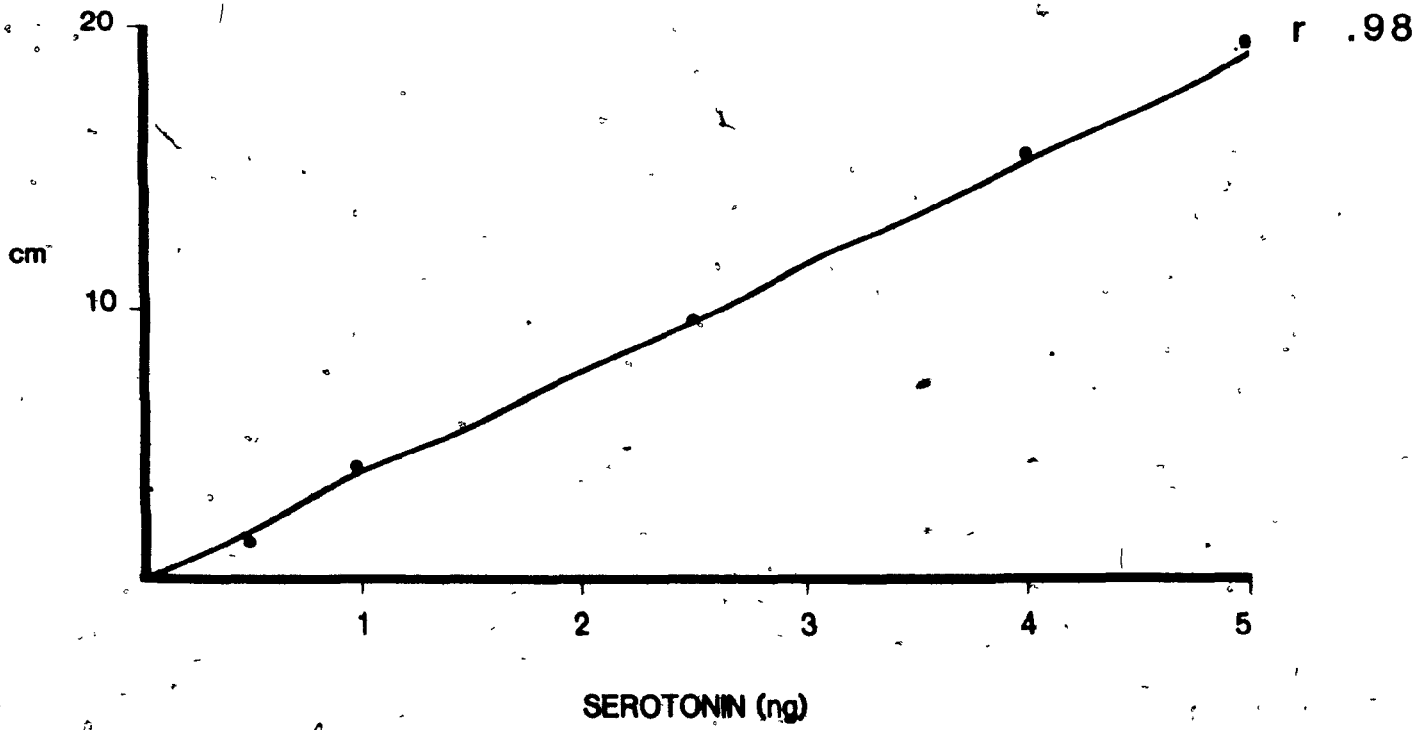


Figure 6

Linear correlation of standards of serotonin.

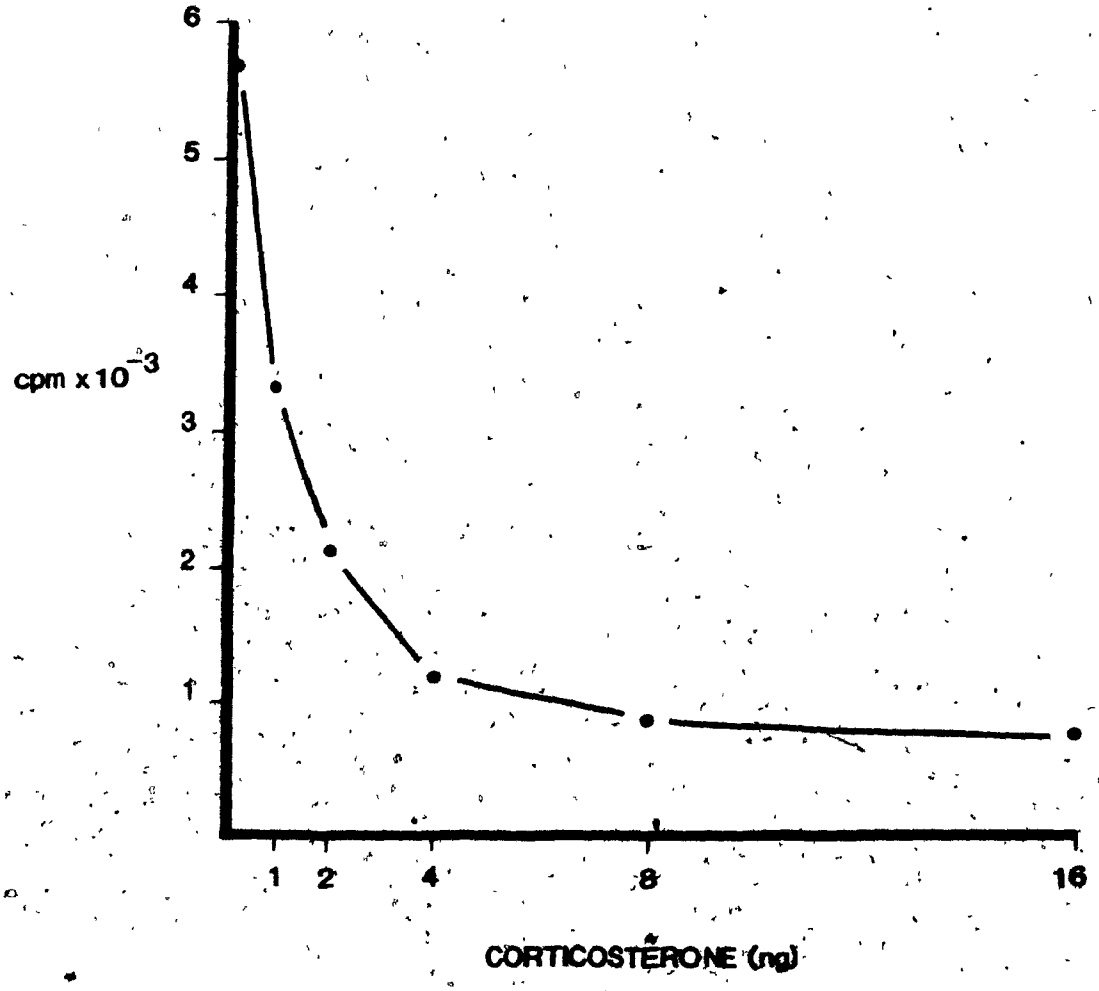


Figure 7

Representative standard curve of corticosterone assay

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3.- RESULTS AND DISCUSSION

3.B. Purification of bovine adrenal PNMT

See subsection 3.H.2, pp. 82

3.D. Catecholamine levels

3.D.1. Adrenal and brain of reserpine-treated rats

Adrenal CA were determined in reserpine-treated rats. The animals received the depletor in a dose of 2.5 mg/kg ip daily for three days. Brain NA, AD and DA decreased by 82, 79 and 68%, respectively. Adrenal NA, AD and DA were depleted by 65, 64 and 79%, respectively (Figures 1).

3.D.2. Adrenal of alpha-methyl-p-tyrosine-treated rats

In these experiments rats received reserpine, AMPT, or the combination of the two drugs, as was done to test their effects on adrenal DBH activity, and CA were determined. Reserpine was administered ip in a dose of 2.5 mg/kg daily; AMPT, 200 mg/kg, was given ip daily in two doses. Both drugs were injected on three succesive days. Control values are shown in Figure 2. AMPT decreased NA, AD and DA by 68, 38 and 58%, respectively. The combination of the two drugs produced decreases of 83, 59 and 79% in NA, AD and DA, respectively (Figure 2).

3.D.3. Adrenal and brain of rats treated with 6-hydroxydopamine (iv)

6OHDA, 100 mg/kg (iv) daily for two days, was administered to the rats. The animals were sacrifice three days after the last injection. This treatment did not modify adrenal or brain concentrations (Table I).

3.D.4. Adrenal of hemisplanchnicotomized rats receiving insulin

In order to test the degree of denervation of the adrenal gland in the hemisplanchnicotomy operation insulin, 100 IU/kg sc, was

administered to fasted rats and animals were sacrificed 3 h later. Failure of the putative denervated glands to respond to insulin stimulation was observed. Depletion of adrenal CA would have indicated the presence of residual intact fibres to the adrenal (Table III).

3.E. Serotonin levels

3.E.1. Forebrain and raphe area of PCPA and DHT -treated rats

See subsection 3.H.4, pp. 84.

3.E.2. Brain of PCPA, DHT and reserpine-treated rats

5HT was determined in the whole brain of rats receiving PCPA, 300 mg/kg ip; DHT, 175 ug icv; reserpine, 2.5 mg/kg ip daily for three days or the combination of PCPA and reserpine, or of DHT and reserpine.

Five days after the administration of PCPA in the indicated dose, a decrease of 75% in brain 5HT was observed; reserpine produced a depletion of 54%; and the combination of the two drugs depleted central serotonin by 97%. Eight days after the administration of DHT icv a decrease of 49% in serotonin was obtained; the combination of the neurotoxin with reserpine depleted brain serotonin by 89% (Figure 3).

3.F. Induction of adrenal DBH by reserpine and oxotremorine

3.F.1. Reserpine and the dose-dependent increase in DBH activity

See Figure 1, subsection 3.H.4, pp. 82.

3.F.2. Kinetics of the enzyme in control and induced forms

Kinetics experiments were carried out with adrenal DBH preparations from control and reserpine treated rats. Lineweaver-Burk plots are shown in Figure 4. Reserpine increased DBH activity by 90%. The Km for the substrate tyramine in control rats was 0.256 mM and in reserpine-trated rats, 0.243 mM (Figure 4).

Adrenal glands of control and oxotremorine-treated rats were also

used for kinetic experiment. The supernatant of a homogenate prepared from 4 adrenal glands was dialyzed against phosphate buffer, 0.02 M, pH 6.8, for 18 h. Figure 5 shows that the value of Km for control, 0.11 mM and for oxotremorine-treated rats, 0.09 mM.

3.F.3. Blockade of reserpine and oxotremorine effects by cycloheximide

In order to test if the blockade of translation by cycloheximide could affect the inducing effect of reserpine and oxotremorine, two experiments were carried out in which reserpine and oxotremorine were given alone or in combination with cycloheximide as specified in Table III. Cycloheximide abolished the effect of the inducers, but it did not have any effect on adrenal DBH activity when given alone.

3.G. Adrenal dopa decarboxylase and lactate dehydrogenase activities in reserpine- and oxotremorine-treated rats

The specificity of the inducing effect of reserpine and oxotremorine on adrenal DBH was tested by the determination of two other adrenal enzymes: dopa decarboxylase, the enzyme that converts DOPA to DA and, for this reason, one that is related to catecholamine biosynthesis, and lactate dehydrogenase, an enzyme not directly involved in monoamine synthesis. Reserpine and oxotremorine, given according to protocols that result in increased adrenal DBH activity, did not alter adrenal dopa decarboxylase or lactate dehydrogenase activities (Table IV).

Table I

Effect of the intravenous administration of 6OHDA on adrenal and brain catecholamine concentration.

Treatment	Noradrenaline	Adrenaline	Dopamine
Adrenal (µg/gland)			
Control	4.63 ± 0.78	9.86 ± 0.78	0.34 ± 0.01
6OHDA	4.38 ± 0.51	8.43 ± 0.45	0.30 ± 0.01
Brain (ng/g)			
Control	2.21 ± 0.40	0.24 ± 0.02	8.61 ± 0.40
6OHDA	2.45 ± 0.05	0.27 ± 0.01	8.18 ± 0.10

Each value is Mean ± SE. 6OHDA was given iv in a dose of 100 mg/kg daily for 2 d. Animals were sacrificed 3 d after the last injection.

Table II

Effect of insulin on adrenal noradrenaline and adrenaline of hemisplanchnicotomized rats.

		Intact adrenal		Denervated adrenal	
Treatment	N	NA	AD	NA	AD
		(μg/adrenal)			
Control	3	2.54 ± 0.22	9.08 ± 0.11	2.56 ± 0.21	10.18 ± 0.67
Insulin	4	1.43 ± 0.13[a]	1.20 ± 0.16[a]	2.27 ± 0.18	9.42 ± 0.51

Each value is Mean ± SE. Hemisplanchnicotomy was carried out 4 d prior to insulin administration. Insulin was given in a dose of 100 IU/kg sc to rats fasted overnight. Sacrifice was done 3 h later.

a $P < 0.01$

Table III

Effect of cycloheximide on the induction of adrenal DBH by reserpine and oxotremorine.

Experiment	Treatment	DBH activity (nmoles per 30 min per adrenal) N	Without cycloheximide	N	With cycloheximide
A	Control	4	7.8 ± 0.8	5	6.6 ± 0.6
	Reserpine	4	11.9 ± 0.4[a]	4	8.3 ± 0.5
B	Control	4	9.3 ± 0.9	4	6.1 ± 0.5
	Oxotremorine	3	15.4 ± 1.6[a]	3	7.8 ±0.6

Each value represents Mean ± SE. Experiment A: Reserpine was given in a dose of 10 mg/kg ip once on day one. Cycloheximide was given, 1 mg/kg ip 1 h after reserpine on day 1 and 0.5 mg/kg ip daily on days 2, 3 and 4. Animals were sacrificed 96 h after reserpine injection. Experiment B: Oxotremorine was given in a dose of 0.5 mg/kg sc twice a day 30 min after 5 mg/kg ip of methylatropine for 4 d. Cycloheximide was given 2 h after each injection of oxotremorine. Sacrifice was done 18 h after the last injection.

a $P < 0.005$ with respect to control

Table IV

Effect of reserpine and oxotremorine on adrenal dopa decarboxylase(DDC) and lactate dehydrogenase (LDH)

Treatment	DDC activity (nmoles/mg protein)	LDH activity (Units/mg)
Control	8.2 ± 0.9	2.8 ± 0.4
Reserpine	8.4 ± 0.7	3.2 ± 0.4
Oxotremorine	7.8 ± 0.9	2.8 ± 0.3

Each value is Mean ± SE. N = 4. Reserpine was given in a dose of 2.5 mg/kg ip once a day for 3 days. Oxotremorine was given in a dose of 0.5 mg/kg sc, 30 min after 5 mg/kg of methylatropine twice a day for 3 d. Rats were sacrificed on the fourth day.

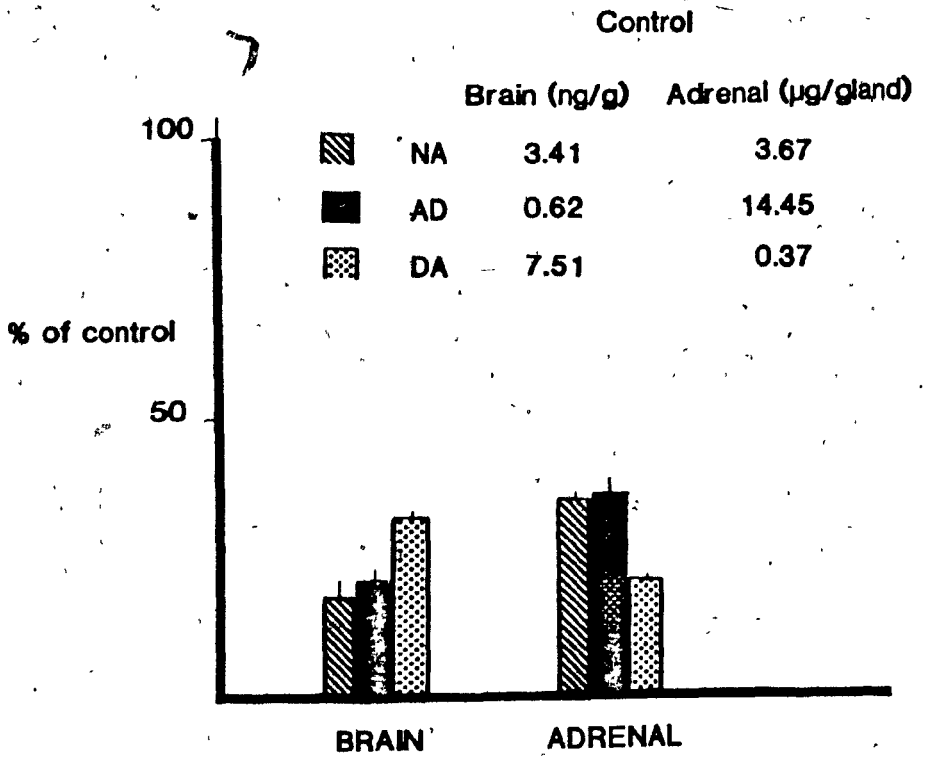

Figure 1

Effect of reserpine on adrenal and brain catecholamines.

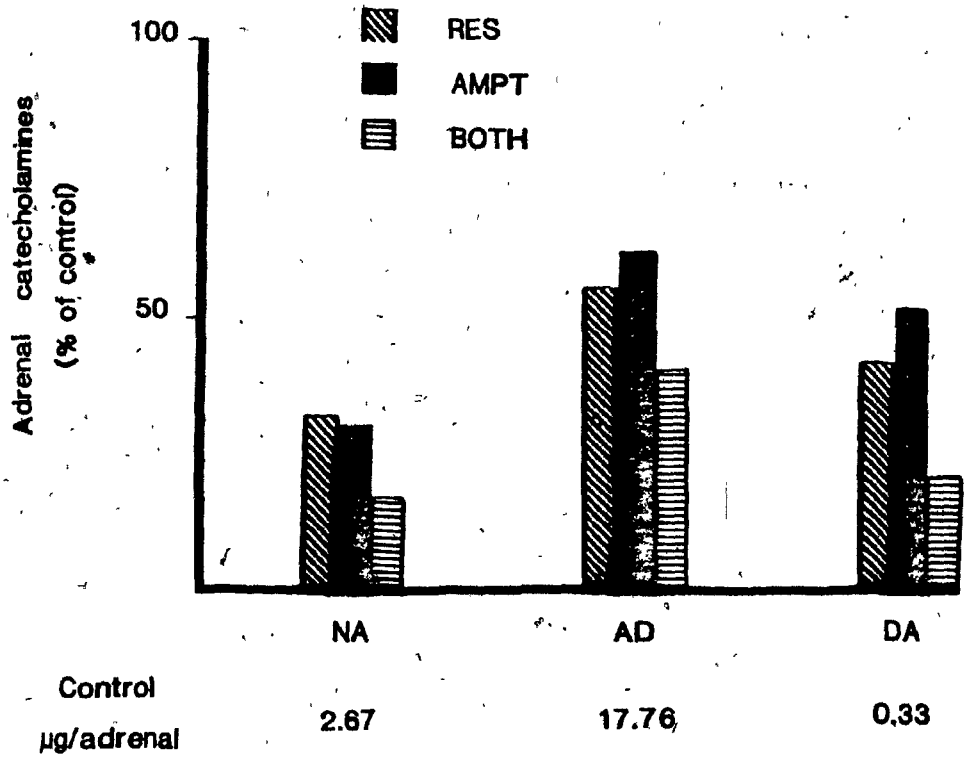

Figure 2

Effect of reserpine and AMPT on adrenal catecholamine concentration.

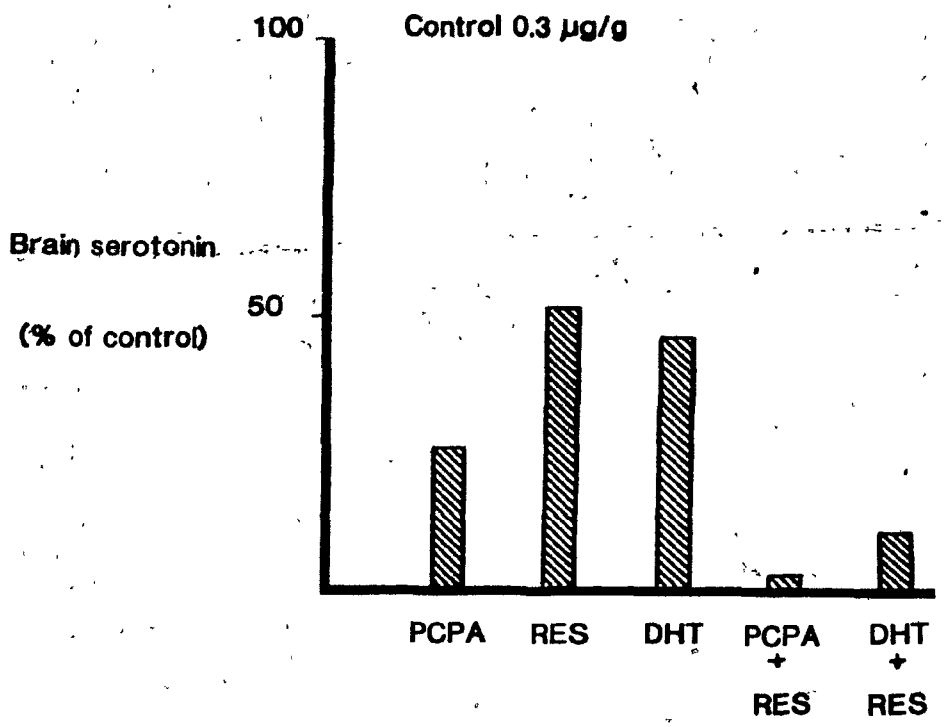

Figure 3

Effect of reserpine, PCPA and DHT on brain serotonin concentration.

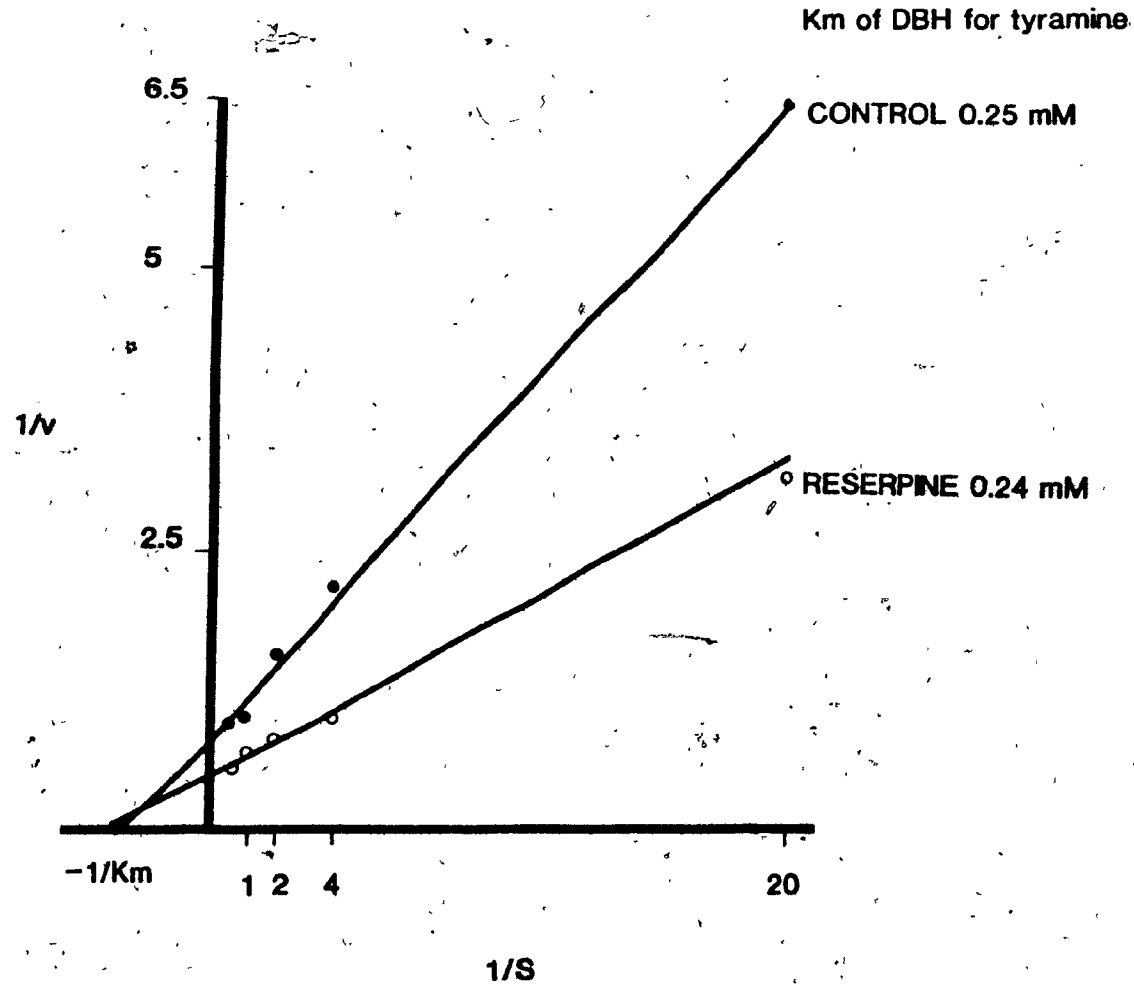

Figure 4

Lineweaver-Burk analysis of adrenal DBH in control and reserpine-treated rats.

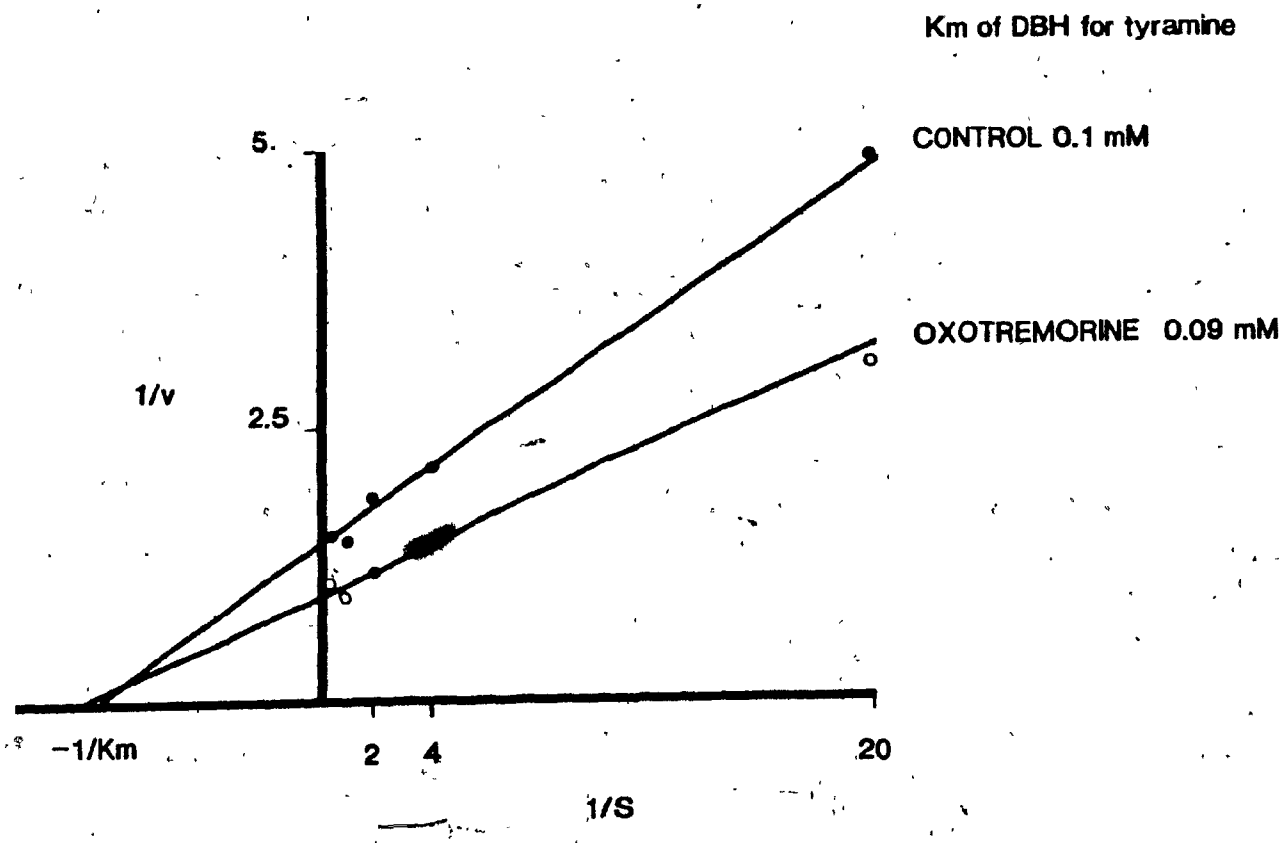

Figure 5

Lineweaver-Burk analysis of adrenal DBH in control and oxotremorine-treated rats.

In accordance with the requirements of the Thesis Examining Committee, pages 69 to 76 of the submitted thesis have now been omitted or the information contained therein has been placed elsewhere.

3.H. Reserpine and the monoaminergic regulation of adrenal DBH activity

3.H.1. Abstract

The systemic administration of reserpine to rats increases adrenal dopamine beta-hydroxylase activity, but is without significant effect on the K_m (tyramine). This induction is partially blocked by hemisplanchnicotomy and by impairment of translation. The combined administration of (a) alpha-methyl-p-tyrosine and p-chlorophenylalanine; (b) 6-hydroxydopamine and p-chlorophenylalanine; or (c) alpha-methyl-p-tyrosine and 5,7-dihydroxytryptamine increases adrenal dopamine beta-hydroxylase activity. These results suggest that the simultaneous depletion of central serotonin and catecholamines, as achieved by reserpine alone or by conjoint action of two specific drugs, is necessary for the induction. p-Chlorophenylalanine (ip) or 5,7-dihydroxytryptamine (icv or injected into the MRN) increases the effect of reserpine, but the use of a catecholamine-depleting agent with reserpine does not alter the increase of adrenal dopamine beta-hydroxylase obtained with reserpine alone. The potentiation by 5,7-dihydroxytryptamine is abolished by hemisplanchnicotomy, a result that demonstrates neural mediation of its effect. Although intravenous administration of 6-hydroxydopamine alone increases the activity of adrenal dopamine beta-hydroxylase, the combination of this treatment with p-chlorophenylalanine does not further elevate it, as occurs with intracerebroventricular injections; this suggests a specific role of central catecholamine depletion. The serotonin agonists 5-hydroxytryptophan, fenfluramine and 5-methoxy-N,N-dimethyltryptamine abolish the inducing effect of reserpine. This work sheds light on the action of reserpine as inducer and provides evidence for the role of monoaminergic pathways, with net inhibitory effects, that are involved

in the regulation of the activity of an adrenal enzyme.

3.H.2. Introduction

Dopamine beta-hydroxylase (DBH, EC 1.14.2.1), the enzyme that catalyzes the conversion of dopamine to noradrenaline,[18] can be induced in the adrenal gland by stressors as well as by certain drugs.[11,28,33] For example, the administration of reserpine to experimental animals causes an increase in adrenal DBH activity[46] that is prevented by denervation of the gland.[11,33] Immunochemical and radiolabeling techniques show that the increased DBH activity is associated with an increase in the rate of synthesis of the enzyme.[11,12,21] The increase is attributed to significant changes in the level of activity of the sympathetic nervous system that result in a postulated reflex increase in activity of the preganglionic (splanchnic) nerves to the adrenal gland.[13,44] Although nerve stimulation brings about an increase in activity of tyrosine hydroxylase, another inducible enzyme, there is as yet no electrophysiological evidence that reserpine actually induces an adrenal enzyme by this means.[51]

In addition to the above problem, there is the related matter of the site (or sites) at which reserpine's action is specifically effective in eliciting the induction of enzymes in the adrenal glands and elsewhere, and the relation of depletion of neuronal monoamines to that induction. Supraspinal cholinergic, dopaminergic and serotonergic systems have been detected as playing roles in the induction of other adrenal enzymes,[42] so it was considered worthwhile to investigate some of these in regard to reserpine's induction of adrenal DBH activity. In this work special attention has been directed toward the depleting action of reserpine on central stores of serotonin and catecholamines.

3.H.3. Materials and Methods

Drugs. Reserpine, serotonin, 5,7-dihydroxytryptamine creatinine sulfate (DHT), 6-hydroxydopamine hydrochloride (6-OHDA), tyramine HCl, DL-p-chlorophenylalanine methyl ester HCl (PCPA), alpha-methyl-DL-p-tyrosine methyl ester (AMPT), 5-hydroxytryptophan (5HTP) and 5-methoxy-N,N-dimethyltryptamine were purchased from Sigma Chemicals, St. Louis, MO; and chloral hydrate, USP, from Fisher Scientific Co., Montreal, Quebec. Fenfluramine was a gift of A.H. Robins, Canada. Imipramine and desipramine were donated by Geigy Canada Ltd. Doses of the neurotoxins are given as the weight of the salt injected into the lateral ventricles.

Animals. Male Sprague-Dawley rats weighing 200 ± 10 g were used in the non-operated groups. Animals submitted to surgery weighed 150 ± 20 g initially. They were obtained from Canadian Breeding Farms and Laboratories Ltd., St. Constant, Quebec. Hypophysectomized animals were also obtained from this supplier and were used 8-11 days after operation. None of the animals designated for hypophysectomy gained weight during this period in the laboratory. Rats were kept in an animal room with a light-dark cycle of 12 hours. The animals were fed ad libitum with Purina Checkers. They were in individual cages at least one day prior to experiment. Control animals received vehicle by the same route as the experimental group and also received the same number of injections.

Surgery. Hemisplanchnicotomy was done under chloral hydrate anesthesia, 300 mg/kg ip, 4 days prior to the start of the experiments. The tissues around the adrenal were completely dissected, except for protection of the vascular supply of the gland. The mean weights of the adrenals on the denervated and intact sides were not significantly

different. The efficiency of the denervation was tested by giving insulin, 100 IU/kg sc, to fasted rats: depletion of catecholamines indicates intact innervation. Adrenals denervated in the manner described did not respond to insulin stimulation.

The coordinates for the intracerebroventricular (icv) injections were L 1.5, P 1 and V 3.5 mm; and for injections in the dorsal raphe nucleus (DRN) and medial raphe nucleus (MRN) they were[26] A (-)350 μm, V (-)0.6 mm and A (-) 350 μm, V (-) 2.6 mm, respectively. Craniotomy was performed at the site corresponding to the place of injection. The ventricular target site was confirmed by injection of methylene blue: the brain of such animals was then sectioned transversally to verify the presence of methylene blue in the cavities. A Hamilton syringe with 26-gauge needle was used for the injection of the neurotoxin. Sham-operated controls received only the vehicle. The injection was performed over the course of one minute, in order to avoid asymmetric uptake.[6] A dose of 175 μg DHT was administered in 20 μl of 0.1% ascorbic acid in saline. A square of Gelfoam was placed over the burr-hole in the bone, and the skin was closed with a metal clip.

Tissue. Animals were deeply anesthetized with Brietal (sodium methohexital), 65 mg/kg, and then the adrenal glands were removed and placed on ice. The capsule and fat were removed and the glands were weighed. Homogenization was done with a Teflon homogenizer in 1 ml of 0.05 M Tris buffer pH 7.4 containing 0.1% Triton X-100. Homogenates were centrifuged at 10,000 g for 10 min. Supernatant was kept at -70° C for up to 4 days prior to assay.

DBH assay. DBH was determined by the two-step assay of Molinoff et al.[34] Blanks were prepared with substrate omitted, with boiled enzyme,

or with 1 mM fusaric acid added; these did not vary significantly. Each sample was prepared in duplicate, variation between them being 1-5%. An internal standard of 50 ng of octopamine was included in all experiments.

PNMT purification. Phenylethanolamine N-methyltransferase (PNMT, EC 2.1.1.28) was partially purified from bovine adrenal medulla according to Díaz Borges et al.[16] Approximately 10 g of tissue were homogenized in 10 volumes of 50 mM phosphate buffer, pH 7.4, containing 0.15 M KCl, 1 mM EDTA and 0.1 mM dithiothreitol. The homogenate was centrifuged at 100,000 g for 1 hour. The supernatant was submitted to salt fractionation with ammonium sulfate from 0-40% (precipitate discarded) and from 40-55%. The second precipitate was collected by centrifugation, resuspended and dissolved in 1 mM phosphate buffer, pH 7.4, containing 1 mM EDTA and 0.1 mM dithiothreitol. The solution was dialysed for 12 h against the resuspending solution. Individual portions of the dialysed preparation (30 ml) were stored frozen at -70°C. There was little loss of enzyme activity for at least up to 6 months. The specific activity of the dialysed solution is 4 nmoles of synephrine formed from octopamine h^{-1}(mg of protein)$^{-1}$. The dialysed preparation was applied to a Sephadex G-200 column (90 x 2.6 cm) previously equilibrated with 0.1 mM dithiothreitol, 1 mM EDTA, 40 mM KCl and 1 mM phosphate buffer, pH 7.4. Elution was done with the same solution. Fractions with enzymatic activity were pooled, lyophilized and resuspended. The specific activity after this step is 35 nmoles of synephrine formed h^{-1}(mg protein)$^{-1}$. (Table IX).

Serotonin and catecholamine determinations. Serotonin was determined by reversed-phase high performance liquid chromatography (HPLC) with electrochemical detection, according to the method of

Anderson et al.[4] The forebrain and raphe nuclei area were dissected according to the procedure of Aghajanian et al.[1] Catecholamines were also determined by reversed-phase HPLC with electrochemical detection by the method of Felice et al.[17]

Statistical analysis. Results are expressed as mean ± standard error. Significance of differences between mean was calculated by Student's t-test. Analysis of variance[49] was carried out in indicated experiments.

3.H.4. Results

Effect of reserpine on the DBH activity of the denervated adrenal gland. Earlier investigators have shown that the daily administration of reserpine to rats brings about significant increases in the activity of adrenal DBH,[11,33] and that this effect is blocked by denervation of the gland.[11] In the present work two experiments with hemisplanchnicotomized rats were performed. As the results were quantitatively similar the data have been pooled, as shown in Table I. Transection of the left splanchnic nerve did not modify the resting activity of adrenal DBH, as compared with the sham-operated controls, in agreement with the earlier reports,[11] nor was the weight of the adrenal glands affected (data not shown). There was a significant increase of DBH activity in the denervated adrenals ($P < 0.05$), owing to reserpine administration, despite the lack of neural input, but this increase was smaller than that produced in the intact gland ($P < 0.05$). Additional results supporting these experimental conclusions are presented later (Figs. 2 and 3).

The nature of the increased activity with reserpine was examined in related experiments. In one of these the K_m of DBH for tyramine was

determined in control and reserpine-injected rats, the latter receiving 2.5 mg/kg ip. For this purpose the adrenal glands were homogenized and the supernatant fraction obtained by centrifuging the homogenate for 0.5 h at 10,000 g was dialysed for 10 h. The K_m values were 0.26 and 0.24 mM for control and reserpine-treated rats, respectively. Cycloheximide, 1 mg/kg daily, blocked the increase of adrenal DBH activity produced 96 h after a single injection of 10 mg/kg of reserpine.

Reserpine was also administered to rats hypophysectomized 8 to 10 days prior to the beginning of the treatment. In sham-operated animals reserpine increased the activity of DBH from 8.3 ± 0.9 nmoles per 30 min per adrenal (Controls, N = 7) to 12.1 ± 1.3 (Reserpine-treated group, N = 6). A similar increase was observed in hypophysectomized rats: from 7.2 ± 0.7 (N = 6) to 12.3 ± 1.3 (N = 6). The resting activity of adrenal DBH was not altered by the hypophysectomy.

Effect of PCPA and DHT on serotonin brain levels. The content of serotonin in the raphe area and forebrain was determined in rats treated with PCPA and DHT (Table II). Four days after the systemic administration of 300 mg/kg of PCPA decreases of about 70% in serotonin levels in both brain areas were observed. The icv administration of DHT depleted serotonin by 43% in the forebrain and by 37% in the raphe area (Table II). Reserpine, 2.5 mg/kg for three days produced a depletion of 45-64% of brain serotonin. The combination of reserpine and PCPA ip or DHT icv, given in the described ways, produced decreases in brain serotonin of 95% and 86%, respectively (data not shown).

Effect of reserpine, AMPT and 6-OHDA iv on adrenal catecholamines. The administration of reserpine to rats for three days produced a dose-dependent decrease in adrenal catecholamines (Fig. 1). At the same time there was a dose-dependent increase of adrenal DBH activity. The

administration of AMPT, 200 mg/kg per day for three days, produced decreases of 68, 38 and 48% in adrenal noradrenaline, adrenaline and dopamine (data not shown). This treatment given in combination with reserpine, 2.5 mg/kg daily, produces decreases of 83, 59 and 78% in noradrenaline, adrenaline and dopamine respectively. The administration of 6-OHDA, 100 mg/kg iv daily for two days, as shown by Kostrzewa and Jacobowitz[27] did not alter adrenal catecholamine levels.

Effects of PCPA on the DBH response to reserpine in hemisplanchnicotomized rats. Although reserpine causes large losses of serotonin from the brain it does not affect the synthesis of that amine. Hence, it was of interest to determine if a decrease of serotonin stemming from inhibition of its synthesis would contribute to the induction of DBH. PCPA, an irreversible inhibitor of tryptophan hydroxylase,[25] was given to hemisplanchnicotomized rats in a single dose of 300 mg/kg 24 h before the first of three daily injections of reserpine. PCPA when given in this way decreased serotonin in the raphe area and forebrain by about 70%. The results (Fig. 2) show that PCPA caused an increase of DBH activity in the innervated gland, although this effect was not consistently obtained in similar experiments carried out later. As demonstrated by the results of Fig. 2, reserpine also caused significant increases in DBH activity: a mean increase of 120% in the intact gland ($P < 0.001$) and 54% in the denervated organ ($P > 0.05$). Moreover, the increase in the intact gland was significantly greater than in the denervated one ($P < 0.05$). In animals receiving both PCPA and reserpine a very great potentiation of the induction of the enzyme in both glands obliterated this effect of denervation ($P < 0.001$).

Effect of DHT on adrenal DBH activity and its induction by reserpine. The neurotoxin DHT given to rats icv causes a long-lasting decrease of serotonin in the brain.[6,39] In our experiments, four days after the injection of DHT serotonin was depleted by 43% in the forebrain and 37% in the raphe area. As shown by the results in Fig. 3, there was no significant effect of DHT by itself on adrenal DBH activity. In hemisplanchnicotomized animals receiving a series of reserpine injections on three consecutive days (see legend to Fig. 3) there was an additional increase of DBH activity in the intact adrenal with the administration of DHT but, unlike the case with PCPA, the increase of enzyme activity in the denervated adrenal owing to reserpine was sustained, but not potentiated by DHT (Fig. 3). Thus, the potentiation of the action of reserpine by DHT with respect to adrenal DBH activity depends upon intact innervation of the gland.

In order to eliminate a possible action of DHT on noradrenaline-containing neurons,[5] this neurotoxin was given icv to rats 30 min after they had received desipramine (20 mg/kg, ip). The reserpine treatment of these rats (N = 5) resulted in a mean increase of 140%, as compared to a 60% increase over controls in animals receiving only reserpine and desimipramine (N = 4). The abiding potentiation of reserpine by DHT in these rats makes it unnecessary to attribute any role in this phenomenon to central noradrenergic fibres because the potentiation is still observed if the uptake of DHT into central noradrenergic neurons is prevented by desimipramine treatment.

DHT was tested by injection into two other sites, viz. the MRN and the DRN, in intact rats. The neurotoxin did not affect the endogenous adrenal DBH activity in either case, but the results demonstrate (Table III)that DHT instilled in the MRN is as efficient as icv administration

in potentiating the effect of parenterally administered reserpine on adrenal DBH activity. In sharp contrast to this, there was no influence of the neurotoxin injected into the DRN. Thus, it appears that a pathway involving the MRN, among other sites, is important in the effect of reserpine on adrenal DBH. This pathway is presumably serotonergic.

Effect of AMPT on adrenal DBH activity. In the above experiments reserpine was tested in animals whose cerebral serotonin had first been made to undergo some decrease through use of PCPA or DHT, in addition to that caused by the reserpine treatment itself. To study the parallel situation with regard to the catecholamine content of the brain, rats were given AMPT alone or in combination with reserpine. Thus, this inhibitor of tyrosine hydroxylase[43] could exert its specific depleting action on cerebral catecholamines and favour even further the loss of these substances brought about by reserpine. There was considerable variation in response to AMPT so that the observed mean increase of DBH activity in rats given this drug (Table IV) did not attain statistical significance ($P > 0.05$). When AMPT was combined with reserpine, the influence of the latter drug on DBH activity persisted (Table IV).

AMPT was also given to rats that received PCPA. In this particular experiment neither of the two substances had a significant effect on adrenal DBH activity. However, the simultaneous action of the drugs, entailing decreases in both catecholamines and serotonin levels, through impairment of the corresponding synthetic pathways, produced increases (Table IV, Expt. B) of 70% in adrenal DBH over controls and those animals receiving only AMPT ($P < 0.001$), and 35% over the group receiving PCPA alone ($P < 0.025$). In another experiment AMPT was given after the icv administration of DHT. Again there was no significant

effect with either of the two drugs given alone, but their combination produced a great increase ($P < 0.001$ by comparison with the other means) in adrenal DBH activity (Table IV, Expt. C). The results of these experiments combining the administration of depletors of catecholamines and serotonin are thus consistent with the view that simultaneous decrease of these monoamines is crucial to the induction of adrenal DBH activity.

Effect of 6-OHDA on adrenal DBH activity. 6-OHDA, a neurotoxin affecting the terminations of catecholamine-containing neurons,[45] was injected icv into rats in two similar experiments (Table V), except that in one of them its uptake into noradrenergic and serotonergic terminals was prevented by the pretreatment of the animals with imipramine.[40] The results were qualitatively identical: in neither case did 6-OHDA have a significant effect on adrenal DBH activity, nor did this substance modify the action of reserpine (Table V).

Although, as mentioned earlier, PCPA does not have a consistent effect upon adrenal DBH (Fig. 2; Table IV), it was important to determine whether the deleterious action of 6-OHDA on central catecholaminergic neurons might evoke some change when combined with the lowering of cerebral serotonin caused by PCPA. The results in TableVI indicate that 6-OHDA injected into the ventricles prior to PCPA did have such an effect, more than doubling the increase of activity of DBH over that expected from the two substances acting individually (each value corrected for control mean). 6-OHDA was also tested by the iv route. In this case, the neurotoxin caused a significant increase in DBH activity ($P < 0.001$), as previously reported by Brimijoin (1971),[7] but there was only an additive effect from PCPA given at the same time (Table VI).

Effect of serotonin agonists on adrenal DBH activity. It was reasoned that if reduction of central serotonin stores is important in the action of reserpine in inducing adrenal DBH activity, the increase of serotonergic function by means of serotonin agonists might prevent reserpine from bringing about this change. This hypothesis was tested in a series of experiments with fenfluramine, a serotonin-releasing drug,[14,19,32] and 5-methoxy-N,N-dimethyltryptamine,[19,20,41] a serotonin agonist. Although the experiments were of a homogeneous 2 x 2 design the essential comparison for the test of the hypothesis was of the mean for treatment with both reserpine and agonist and the mean for reserpine alone. In this test (Table VII) both fenfluramine and the tryptamine derivative reduced the response of the adrenal DBH activity to reserpine significantly ($P < 0.025$ and $P < 0.0125$, respectively, by the one-tailed t-test).

The actual cerebral content of serotonin can be increased by administering its precursor to rats previously treated with a peripherally acting inhibitor of aromatic amino acid decarboxylase. This was tested with rats given 5HTP along with carbidopa (Table VIII). The inhibitor did not affect adrenal DBH activity nor the action of reserpine in that regard. The combination of inhibitor and precursor also had little or no effect upon DBH activity. However, when this combination was given to reserpinized rats, the expected increase of DBH activity was no longer in evidence (Table VIII).

3.H.5. Discussion

The increases in adrenal DBH activity obtained in the present work by treatment of rats with reserpine for three days are highly significant, a result in accord with previous reports.[11,33] The use of radiolabeling and immunological techniques has shown that the action of

reserpine is to increase the number of molecules of the enzyme through an increase in the rate of synthesis,[11,13,50] an inductive effect mediated through the splanchnic nerve.[11] Although reserpine is reported to modify splanchnic activity in some way,[51] that mechanism is not well understood. Despite this, our experience in working with unilaterally splanchnicotomized rats receiving reserpine is that an increase of adrenal DBH activity persists even after denervation of the gland, but the increase is significantly smaller than that observed in the intact gland (Table I). This makes it likely that there is a humoral or a peripheral component to the action of reserpine. Indeed, a decrease of cerebral monoamine content caused by reserpine could affect the function of peptidergic neurons in the hypothalamus[35,47] and, consequently, of hypophyseal function. In addition, reserpine is a potent stimulator of ACTH secretion in rats,[29] probably through an increase in the release of corticotropin releasing factor.[8] Thus, in the absence of the main inductive mechanism, i.e. the peripheral neural component, as a result of adrenal denervation, reserpine would produce an endocrine-mediated increase in adrenal DBH activity. A hormonally mediated increase of adrenal enzyme activity is also evident in the case of adrenocortical ornithine decarboxylase.[38] The persistence of the effect of reserpine on adrenal DBH in hypophysectomized rats, as previously reported,[11,48] could then be attributed to the peripheral neural mechanism or, possibly, to a local action.

Reserpine depletes adrenal catecholamines in a dose-dependent manner, and also brings about an increase of adrenal DBH (Figure I). Viveros et al.[46] showed that in the rabbit the neurogenic stimulation of the adrenal medulla produced by reserpine depends on the amount of drug

administered. Doses of 1 mg/kg, unlike higher doses, did not stimulate the release of catecholamines and DBH from the chromaffin granules, but did increase adrenal DBH activity[46] (Figure 1). As has been shown in the rat, the presence of an intact nerve supply is not necessary for the depleting action of reserpine on the adrenal medulla.[9,22] Thus, in order to assure not only a local action of reserpine on adrenal DBH, but also a neurogenic stimulation, a dose of 2.5 mg/kg was used in all the experiments. It has been previously reported[11,48] that the activity of DBH in the adrenal gland of chronically hypophysectomized animals (i.e. more than two weeks following operation) is reduced significantly below that in sham-operated rats. We did not obtain this effect (see Results section), perhaps because in our experiments sacrifice was carried out only 8-11 days after the operation, not long enough to produce a significant biological effect.

Because reserpine causes the loss of many monoamines from the brain it was important to determine the effects of depletion of particular members of this group. The first drugs tested were the serotonin depletors PCPA and DHT. The latter, after icv injection, is taken up into axons and nerve endings[6] where it causes the destruction of these elements, with a result equivalent to serotonergic denervation. In our work DHT caused a 40% decrease in central serotonin content (see Results section), but did not modify adrenal DBH activity (Fig. 3; Tables III and IV). However, there was occasionally a small, increase of DBH in the adrenal of animals receiving PCPA, (Fig. 2; Tables IV and VI). This is quite different from the case of tyrosine hydroxylase induction, where depletion of cerebral serotonin leads to an unequivocal increase in activity.[37] However, the result is not unlike the effect of serotonin depletion on adrenomedullary ornithine decarboxylase.[3]

It is generally accepted that the decrease in monoamines produced by reserpine through blockade of reuptake of these monoamines into storage vesicles[10] is a crucial step in the induction of DBH. The decrease in monoamines, however, is not complete, because their synthesis continues during the time that the animal is in the reserpinized state, and because the enzyme undergoes synthesis in the adrenal. Newly synthesized amines, unable to be retained, would be continually available for binding to receptors and action on appropriate neurons. For this reason an experiment was carried out in which some rats given reserpine also received PCPA to block specifically the synthesis of serotonin. In this case PCPA potentiated the inducing effect of reserpine by 47% (Fig. 2). The fact that this potentiation was not abolished by denervating the adrenal gland, supports the existence of a humoral component that is evoked by PCPA. It is well established that serotonergic inputs affect peptidergic neurons in the hypothalamus, so that a decrease of serotonin might trigger the production of corticotropin releasing factor, adrenocorticotropin and glucocorticoids in succession by eliminating a net negative input. It has been reported[15] that the synthesis of serotonin is more susceptible to inhibition by PCPA in the terminals than in the perikarya of raphe neurons. Continuing synthesis in the raphe cell bodies could then be responsible for the persistence of PCPA effects in hemisplanchnicotomized animals. A peripheral effect could also play a role.

When reserpine was administered to rats that had already received DHT, its effect was potentiated by 53-87%. This increase was sharply modified by denervation of the adrenal gland, as seen in Fig. 3. The

lower activity of the combination of reserpine and DHT after denervation suggests that descending serotonergic fibres or a central serotonergic loop are implicated. Such a loop would cause a net increase in the function of descending excitatory fibres, leading to the enhanced DBH activity.

Thus, PCPA and DHT, two drugs that reduce central serotonergic function by quite different mechanisms, potentiate the action of reserpine on adrenal DBH activity. However, the potentiating effect of DHT is neurally mediated, whereas that of PCPA is not. PCPA might act by producing a stress reaction in rats, as evidenced by increased serum corticosterone concentrations,[31] but a peripheral effect cannot be excluded. Another possibility is that both agents act centrally but at different sites in the brain. There would then be a diversity in serotonergic pathways that produce the differential effects of PCPA and DHT, as seen in hemisplanchnicotomized rats treated with reserpine.

The increase of adrenal DBH activity in rats given AMPT was considerably greater after central serotonergic denervation by DHT than the increase observed after the administration of PCPA (Table IV). Thus, the decrease of serotonin is not the sole factor involved in the potentiation of reserpine's action in respect to adrenal DBH activity. It is probable that the greater potentiation obtained with DHT is due to the loss of other elements in the damaged neurons (e.g. peptides and other modulators), and that this favours the elimination of a net inhibitory effect in the regulation of adrenal DBH.

Our data indicate that cerebral neurotransmitters other than serotonin must be simultaneously decreased in order to induce adrenal DBH. The use of AMPT, an inhibitor of catecholamine synthesis, does not by itself produce a consistent or significant increase of adrenal DBH

activity (Table IV). However, its administration along with PCPA does produce such an increase; this result supports the evidence already accumulated that both monoamines must be decreased in order to effect an increase in enzyme activity (cf. Tables IV and VI). Because AMPT did not modify the inducing effect of reserpine (Table IV, Series A), catecholamine depletion might be considered to play a secondary role in the process of DBH induction. Yet there is evidence in the present results for the interaction of catecholamine and serotonin systems in DBH induction. Thus, the great increase in adrenal DBH activity observed when DHT was injected into the cerebrospinal fluid, to be followed by parenterally administered AMPT (Table IV, Series C), supports the existence of a central serotonergic pathway with a net inhibitory action over the adrenal gland; however, in this case the decrease in catecholamines by giving AMPT ip is systemic, and a peripheral or local contribution is added to the central effect. Furthermore, the increase of adrenal DBH activity occasioned by the simultaneous administration of PCPA and 6-OHDA (the latter given into the ventricles of the brain) shows (Table VI) that central catecholamine sites must be affected if the depletion of serotonin is to produce a marked effect on the adrenal enzyme. It is noteworthy that when this experiment was repeated with iv administered 6-OHDA, a measure that does not deplete brain or adrenal catecholamines, there was no increase of DBH. As 6-OHDA given iv does not enter the brain, the result is a further confirmation of the need of central catecholamine-serotonin interaction in order to effect the induction of DBH. These interactions are shown schematically in Fig. 4. Other such interactions have been detected in the regulation of different biological functions. For example, Jouvet[24] asserts that a

sleep-waking cycle is regulated by two interacting ascending systems, adrenergic and serotonergic. The simultaneous decrease of serotonin and cerebral catecholamines is necessary for facilitation of the hippocampal kindling formation.[2] Biochemical studies have shown that the depletion of cerebral noradrenaline by inhibition of cerebral DBH is accompanied by an increase in the rate of serotonin synthesis.[23]

In order to verify the important role of the decrease of cerebral serotonin in the DBH-inducing action of reserpine, three serotonin agonists were tested to determine whether they are able to overcome that efect (Tables VII and VIII). Fenfluramine, 5-methoxy-N,N-dimethyltryptamine, and 5HTP (given with carbidopa) all effectively diminished the effect of reserpine on adrenal DBH activity. These results, then, implicate serotonin in the regulation of adrenal DBH through a central inhibitory system (see Model, Fig. 4). The results in this work demonstrate, however, that interference with or interruption of the working of this serotonergic system in the brain is necessary but not sufficient to bring about the increase of adrenal DBH activity, for the simultaneous decrease of catecholamines is important also. The analysis of the central serotonergic component affecting adrenal DBH has revealed, moreover, that, PCPA and DHT, used as serotonin-depleting agents along with reserpine, have different sites of action. More specific studies will be necessary in order to determine the role of descending spinal serotonergic projections as well as of forebrain projections that act on the hypothalamus.[30,36]

Fig. 1. Effect of reserpine on adrenal DBH activity and catecholamine content. Rats received 3 daily injections of reserpine in doses of 0.5, 1 or 2.5 mg/kg ip. Sacrifice was done 18 h after last injection. (●) DBH activity. (○) Noradrenaline. (△) Adrenaline. (□) Dopamine.

* $P < 0.05$

+ $P < 0.01$

ǂ $P < 0.001$

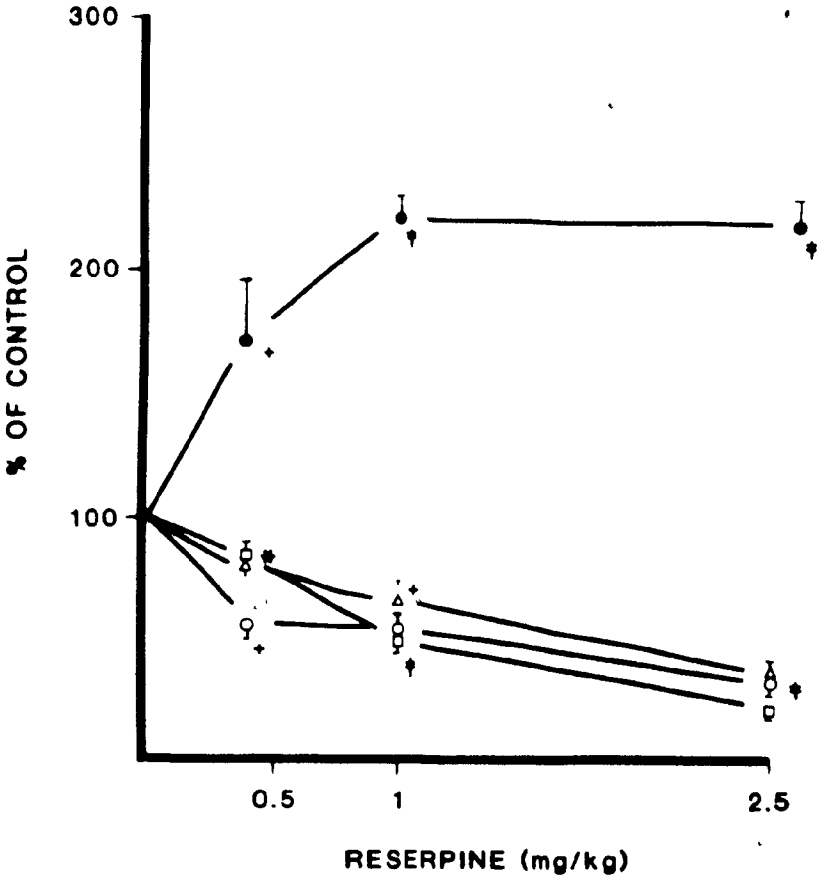

Figure 1

Fig. 2. Effects of PCPA and reserpine on adrenal DBH activity of hemisplanchnicotomized rats. Rats were splanchnicotomized on the left side and allowed to recover from surgery for 4 days. They then received a single ip injection of PCPA, 300 mg/kg. Reserpine, 2.5 mg/kg given ip, was administered at 24, 48 and 72 h after PCPA. The rats were killed 96 h after PCPA administration. The data were subjected to the analysis of variance, with extraction of sums of squares for experiments (1 degree of freedom), innervation (1), treatments (3), interaction of treatments and innervation (3) and remainder (44). Significance of differences between means is based upon the t-test, standard error of differences between means being calculated from the mean square for error (remainder), which was 6.0745. Probabilities for the following comparisons were:

	Innervated gland	Denervated gland
Reserpine vs control	< 0.001	> 0.05
PCPA vs control	< 0.025	> 0.05
Both drugs vs reserpine	< 0.001	< 0.001
Both drugs vs PCPA	< 0.001	< 0.001

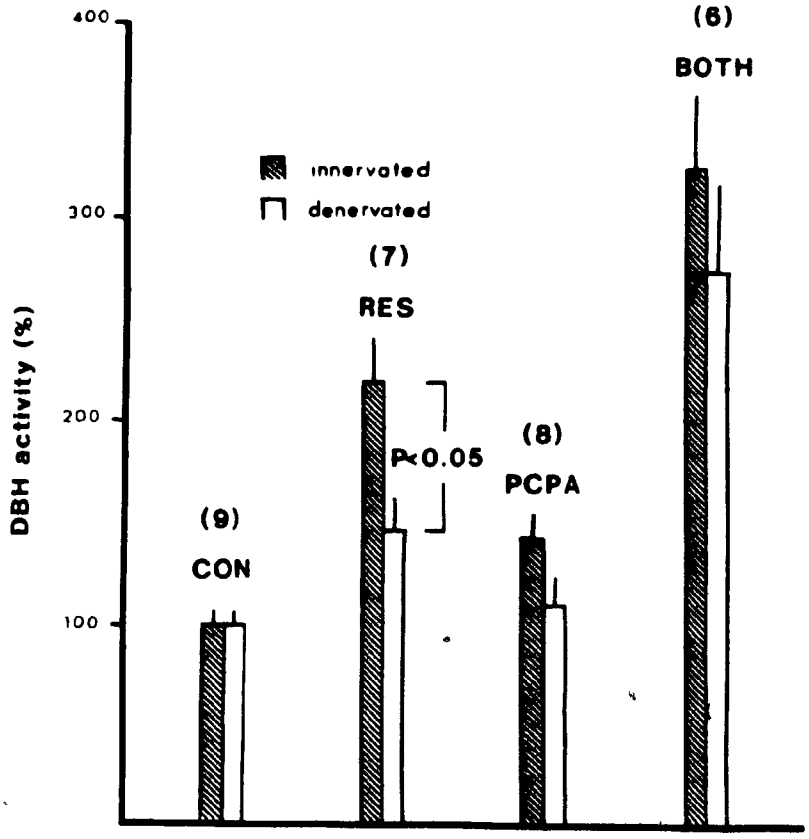
400
300
200
100
DBH activity (%)
innervated
denervated
(9)
CON
(7)
RES
P<0.05
(8)
PCPA
(6)
BOTH

Figure 2

Fig. 3. Effects of DHT and reserpine on adrenal DBH activity of hemi-splanchnicotomized rats. Rats were splanchnicotomized on the left side and allowed to recover from surgery for 7 days prior to the administration of 175 μg of DHT in 20 μl of 0.1% ascorbic acid in saline into the right lateral ventricle during the course of one min. Reserpine, 2.5 mg/kg given ip, was administered for 3 d after beginning on the fourth day after DHT. The rats were killed 24 h after the last injection of reserpine. The data were subjected to the analysis of variance, with extraction of sums of squares for experiments (1 degree of freedom), innervation (1), treatments (3), interaction of treatments and innervation (3) and remainder (35). Significance of differences between means is based upon the t-test, standard error of differences between means being calculated from the mean square for error (remainder), which was 16.0165. Probabilities for the following comparisons were:

	Innervated gland	Denervated gland
Reserpine vs control	< 0.05	> 0.05
DHT vs control	> 0.05	> 0.05
Both drugs vs reserpine	< 0.01	> 0.05
Both drugs vs DHT	< 0.001	< 0.05

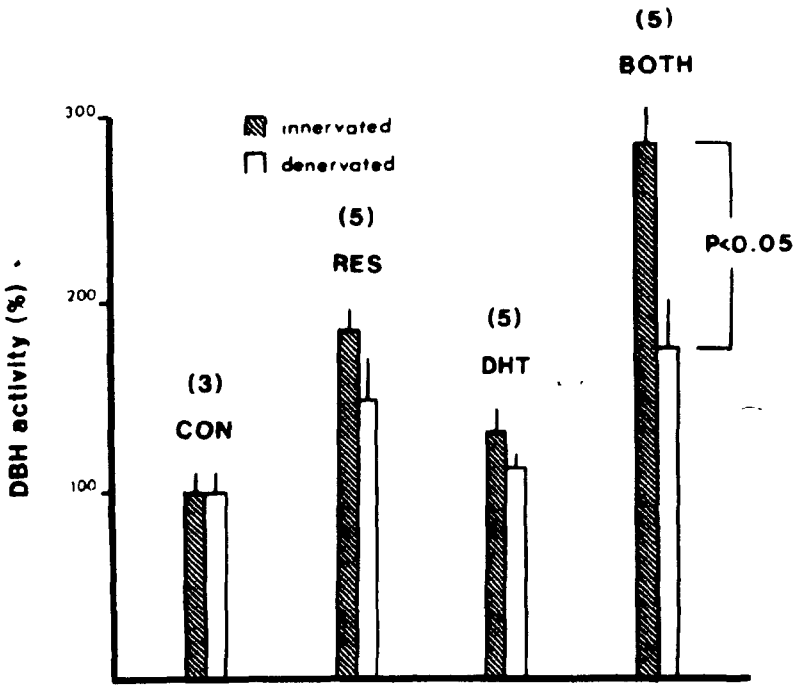
(5)
BOTH
innervated
denervated
300
200
100
DBH activity (%)
(5)
RES
(3)
CON
(5)
DHT
P<0.05

Figure 3

Fig. 4. Role of monoamines in the reserpine-induced increase of adrenal DBH activity: a model.

The specific effects of the drugs on the target organ are indicated by the arrows. (A) Some centres in the central nervous system (CNS) are sensitive to the decrease in catecholamines (CA) and serotonin (5HT) caused by giving reserpine (Table I). One of these centres is the MRN (Table II). The loss of monoamines results in a net decrease of inhibitory outputs from the CNS that are relayed over a neural pathway to the adrenal gland, with a consequent increase of DBH activity in that organ. (B) If these and/or other centres are depleted only of their 5HT content through administration of PCPA or DHT (Figs. 1 and 2), the conditions are insufficient for a significant increase in the activity of adrenal DBH activity. (C) This is also true for specific depletion of CA by administration of AMPT or 6-OHDA (Tables III and IV). (D-E) However, administration of PCPA or DHT together with reserpine imposes additional impairment of 5HT functions in the CNS (Figures 1 and 2), and produces a greater release of net inhibitory outputs than in (A). As a result, there is significantly greater induction of DBH. (F-G) The use of AMPT or 6-OHDA does not potentiate the induction of DBH by reserpine (Table III). (H) The induction of adrenal DBH activity by reserpine is blocked by 5HT agonists (Table VI). Thus, the action of reserpine on DBH activity of the adrenal is exerted primarily through its antiserotonergic effect.

Peripheral effects of reserpine and AMPT, such as the decreases in CA in sympathetic nerve endings, have not been taken into account in this model; they may also be important for the production of the observed adrenal effect. Thus, 6-OHDA acting outside the CNS (Table V)

increases adrenal DBH activity. (I) A peripheral or humorally mediated action of PCPA could also take place, especially considering that its effect in potentiating reserpine action is not abolished in hemisplanchnicotomized rats (Fig. 1).

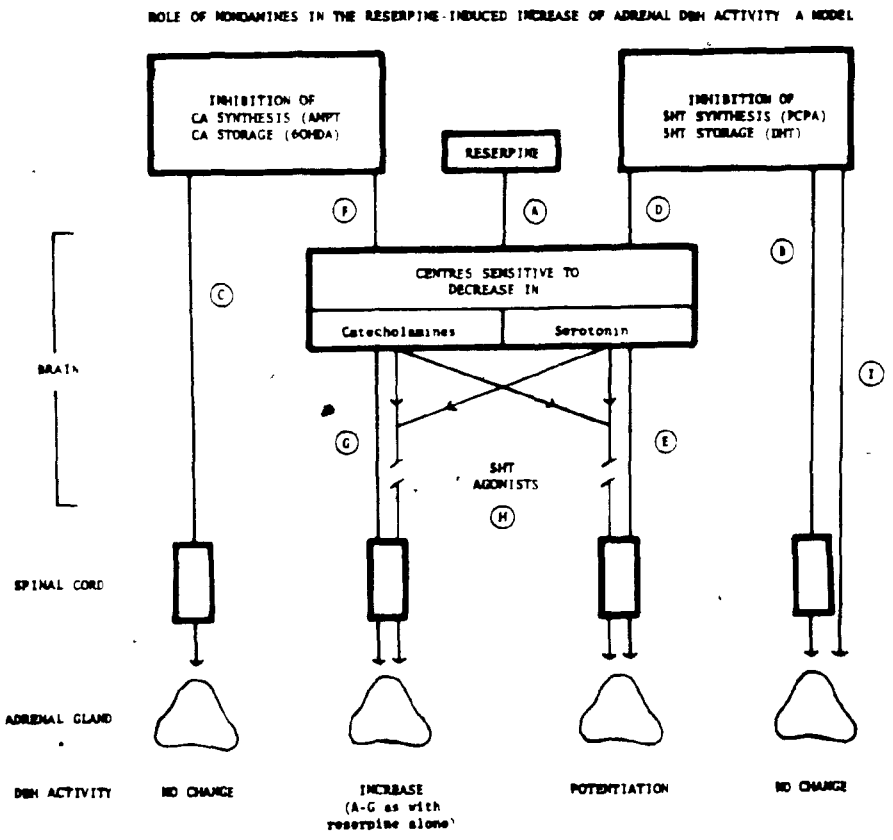
ROLE OF MONOAMINES IN THE RESERPINE-INDUCED INCREASE OF ADRENAL DBH ACTIVITY A MODEL
INHIBITION OF
CA SYNTHESIS (AMPT
CA STORAGE (6OHDA)
RESERPINE
INHIBITION OF
5HT SYNTHESIS (PCPA)
5HT STORAGE (DHT)
F
A
D
B
C
CENTRES SENSITIVE TO
DECREASE IN
Catecholamines
Serotonin
BRAIN
I
G
E
5HT
AGONISTS
H
SPINAL CORD
ADRENAL GLAND
DBH ACTIVITY
NO CHANGE
INCREASE
(A-G as with
reserpine alone)
POTENTIATION
NO CHANGE

Figure 4

Table I. Effect of denervation on the induction of adrenal DBH activity after reserpine

Row	Treatment	N	Adrenal DBH activity (nmoles per 30 min per adrenal) Innervated	Denervated
1	Sham-operated	7	8.2 ± 0.7	7.9 ± 1.1
2	Sham-operated + reserpine	7	16.0 ± 1.9*	15.9 ± 1.9*
3	Left splanchnicotomy	8	8.2 ± 0.5	7.6 ± 0.6
4	Left splanchnicotomy + reserpine	9	14.1±1.8+	11.2 ± 1.9+

Rats were operated on for section of the left splanchnic nerve; sham-operated animals underwent similar surgery, except that the left splanchnic nerve was left intact. Four days after surgery reserpine was administered ip in a dose of 2.5 mg/kg. This was repeated on 3 successive days and the animals were killed 24 h after the last injection. Means ± Standard Error are shown. Because there was denervation of one adrenal, but not the other, in rats belonging to two of the four groups, a split-plot analysis of variance was carried out for the purpose of comparing individual means.[49] The probabilities of the differences between means derived from ANOVA are as follows: *$P < 0.01$ for the effect of reserpine in sham-operated rats (comparison of rows 1 and 2, both adrenals taken into account); +$P < 0.05$ for the effect of reserpine on enzyme activity in denervated adrenals (comparison of rows 3 and 4); $P < 0.05$ for the effect of denervation on the response to reserpine (comparison of rows 2 and 4, denervated adrenal only).

Table II. Effect of PCPA and DHT on brain serotonin content.

Treatment	N	Serotonin (ng/g)	
		Forebrain	Raphe area
Control	4	157 ± 10	258 ± 10
PCPA	3	45 ± 2	80 ± 4
DHT	3	90 ± 1	162 ± 2

Each value represents mean ± standard error. PCPA was given in a dose of 300 mg/kg ip once, 4 d before the sacrifice. DHT was given icv in a dose of 175 μg in 200 μl saline containing 0.1% ascorbic acid 8 d before sacrifice.

Table III. Effects of DHT administered at different sites in the brain and of reserpine on adrenal DBH activity of rats

Site of of DHT administration	Adrenal DBH activity (nmoles/30 min per adrenal)			
	Control	Reserpine	DHT	Both
Lateral ventricle	8.2 ± 0.6	15.1 ± 1.4	8.6 ± 1.3	28.1 ± 5.1
Medial raphe nucleus	7.3 ± 0.7	13.6 ± 1.6	9.2 ± 1.2	28.2 ± 3.6
Dorsal raphe nucleus	11.6 ± 1.2	16.7 ± 2.2	12.8 ± 0.8	16.4 ± 1.4

Rats received DHT, 175 μg in 20 μl of 0.1% ascorbic acid in saline, by stereotaxic injection into the lateral ventricle; others received 10 μg of the compound in 5 μl of 0.1% ascorbic acid in saline by injection into the medial raphe nucleus or dorsal raphe nucleus. Reserpine was given ip in a dose of 2.5 mg/kg daily for 3 d, and the animals were killed 24 h after the last injection. Control means ± Standard Error (estimated from the error variance in analysis of variance) are shown (number of rats per group, 4-9).

Significance of the difference: in each case the mean effect of reserpine (alone or with DHT) is significantly greater than the effect of DHT alone or controls ($P < 0.05$). However, the effect of reserpine in rats that received DHT in the lateral ventricles or MRN was significantly greater than in those receiving vehicle only ($P < 0.05$).

Table IV. Effects of AMPT, reserpine, PCPA and DHT on adrenal DBHactivity

Series	Treatment	N	Adrenal DBH activity (nmoles per 30 min per adrenal)
A	Control	4	7.6 ± 0.6
	Reserpine	4	13.4 ± 0.7
	AMPT	4	11.1 ± 2.0
	Both	4	14.1 ± 1.4
B	Control	4	8.5 ± 0.3
	PCPA	4	10.7 ± 0.8
	AMPT	4	8.5 ± 0.1
	Both	4	14.4 ± 0.8
C	Control	5	9.1 ± 0.2
	DHT	5	9.1 ± 0.4
	AMPT	6	11.6 ± 1.1
	Both	5	24.0 ± 1.1

Each value represents Mean ± Standard Error.

Series A: Reserpine was given in a dose of 2.5 mg/kg ip once daily for 3 d, 2 h after the animals received 200 mg/kg ip of AMPT. Series B: PCPA, 300 mg/kg ip, was given once, 24 h before the first of a series of injections of AMPT; this drug was given in a dose of 200 mg/kg ip twice daily for 4 d. Series C: DHT was administered in a dose of 175 μg in 20 μl of 0.1% ascorbic acid in saline, into the right lateral ventricle. Four days later AMPT injections were begun: 200 mg/kg ip twice daily for 4 d. Animals were killed 18 h after the last injection.

Table V. Effect of 6-OHDA and reserpine on adrenal DBH activity

Treatment	N	Adrenal DBH activity (nmoles/30 min per adrenal)
No pretreatment		
Control	3	5.0 ± 0.4
Reserpine	4	9.9 ± 0.5
6-OHDA	3	4.6 ± 0.3
Both	4	10.5 ± 1.5
Pretreated with imipramine		
Control	4	8.8 ± 0.6
Reserpine	6	12.5 ± 0.8
6-OHDA	5	8.9 ± 0.7
Both	5	13.7 ± 1.0

Each value is Mean ± Standard Error. 6-OHDA was given by stereotaxic injection into the right lateral ventricle in a dose of 200 μg in 20 μl of 0.1% ascorbic acid in saline. Twenty-four hours later injections of reserpine were begun: 2.5 mg/kg ip daily for 4 d. Animals were killed 24 h after the last injection.

Table VI. Effect of p-chlorophenylalanine (PCPA) and 6-hydroxydopamine (6-OHDA) on adrenal DBH activity

Treatment	6-OHDA, icv		6-OHDA, iv	
	N	DBH activity	N	DBH activity
Control	4	7.8 ± 0.6	7	9.9 ± 0.6
PCPA	6	9.3 ± 0.4	3	11.7 ± 0.6
6-OHDA	5	8.2 ± 0.5	6	14.3 ± 0.8*
6-OHDA + PCPA	6	11.8 ± 1.0^{+}	3	17.1 ± 2.0*‡

Each value represents a mean DBH activity ± standard error, in nmoles/ 30 min per adrenal. 6-OHDA icv: 6-OHDA was administered in a dose of 200 μg in 20 μl of 0.1% ascorbic acid in saline, into the right lateral ventricle during the course of 1 min. Four days later PCPA was given by ip injection, 300 mg/kg. Animals were killed 4 d after the PCPA. 6-OHDA iv: Rats received PCPA, 300 mg/kg ip; 24 and 48 h later 6-OHDA was injected iv in a dose of 100 mg/kg. Animals were killed 3 d after the second dose of 6-OHDA.

*$P < 0.001$ with respect to control

$^{+}P < 0.05$ with respect to PCPA

$^{\ddagger}P < 0.05$ with respect to the other means

Table VII. Effect of serotonin agonists and reserpine on adrenal DBH activity

Treatment	DBH activity (nmoles/30 min per adrenal)			
	N	Without reserpine	N	With Reserpine
A Control	6	8.0 ± 0.7	6	11.7 ± 0.4*
Fenfluramine (10 mg/kg)	7	10.2 ± 0.90	7	10.0 ± 0.6^{+}
B Control	4	6.6 ± 0.2	4	10.8 ± 0.3*
5-Methoxy-dimethyltryptamine (1 mg/kg)	4	6.8 ± 0.2	5	8.5 ± 0.8^{+}

Each value represents mean ± standard error. Reserpine was given in a dose of 2.5 mg/kg ip once daily for 3 d preceded 5 h earlier by fenfluramine or 1 h earlier by 5-methoxy N,N-dimethyltryptamine. Rats were killed 24 h after the last injection.

*$P < 0.001$ with respect to control rats without reserpine

$^{+}P < 0.05$ with respect to control rats with reserpine

Table VIII. Effect of reserpine, 5HTP, and carbidopa on adrenal DBH activity

Treatment	N	DBH activity (nmoles/30 min per adrenal)
Control	9	8.1 ± 0.4
Reserpine	9	12.7 ± 0.8
Carbidopa	3	8.2 ± 0.7
Carbidopa + reserpine	3	12.0 ± 0.6
5HTP + carbidopa	7	9.6 ± 1.5
5HTP + carbidopa + reserpine	9	8.5 ± 0.7

Each value respresents mean ± standard error. Reserpine was given in a single dose of 10 mg/kg ip and animals were killed 96 h later. 5HTP was given ip 2 h after the injection of reserpine in a dose of 20 mg/kg, and thereafter twice daily for 4 d. Carbidopa, 25 mg/kg ip, preceded each injection of 5HTP by 30 min. Analysis of variance was done, with extraction of sums of squares for experiments (1 degree of freedom), treatments (3), interaction (3) and error (27). Significance of the differences between means were calculated from the mean square for error (5.4001). Probabilities are as follows: reserpine vs. control, < 0.001; reserpine vs. 5HTP + carbidopa, < 0.025; reserpine vs. carbidopa, < 0.01; reserpine vs. 5HTP + carbidopa + reserpine, < 0.005.

Table IX. Summary of PNMT purification

Fraction	Activity per ml (cpm) x 10^3	Volume (ml)	Total activity (cpm) x 10^3	Protein (mg/ml)	Specific activity (cpm/mg prot) x 10^3	Yield	Fold
Homogenate	212	125.5	26,606	17.8	11.91	100	1
Supernatant (100,000g)	234	121	28,314	3.4	68.82	106	5.77
Ammonium sulfate (0-40%)	123	132.5	20,140	2.09	58.85	76	4.94
Ammonium sulfate (40-55%)	368	50	18,400	1.05	350	69	29.38
Sephadex G-200	36	40	1,440	0.05	720	5	60.45

3.H.6. References

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3.I. Differential role of raphe nuclei in the regulation of dopamine beta-hydroxylase in the adrenal gland of the rat

3.I.1. Abstract

The effect of reserpine on the activity of dopamine beta-hydroxylase (DBH) in the adrenal gland of the rat was determined following electrolytic lesion of the dorsal raphe nucleus (DRN) or medial raphe nucleus (MRN). In sham operated rats, as well as in those with a lesion of the DRN there was no significant modification of the action of reserpine on this enzyme. However, lesion of MRN potentiated the inducing action of the drug. A specific role of MRN in the serotonergic regulation of adrenal DBH is suggested by this work.

3.I.2. Introduction

The administration of reserpine to rats produces a time-dependent increase in the activity of adrenal dopamine beta-hydroxylase (DBH, EC 1.14.2.1) (Molinoff et al., 1970; Ciaranello et al., 1975; Lima and Sourkes, 1985). This induction is potentiated by the conjoint parenteral administration of p-chlorophenylalanine (PCPA) or by the intracerebroventricular injection of 5,7-dihydroxytryptamine (DHT) with reserpine, measures that reduce the amount of brain serotonin to very low levels (Lima and Sourkes, 1985). Although serotonin agonists when given alone do not modify adrenal DBH activity, their administration to reserpinized rats diminishes the increase of DBH observed in such animals (Lima and Sourkes, 1985). It is thus apparent that the antiserotonergic action of reserpine plays an important role in its inducing effect on DBH. Furthermore, the results suggest that a central serotonergic system exerts a net inhibitory influence on adrenal DBH, as has been reported for adrenal tyrosine hydroxylase (TH, EC 1.14.16.2) (Quik and Sourkes, 1977).

Central serotonergic neurons are localized in the midline raphe

nuclei of the brain stem (Dahlström and Fuxe, 1965). These indoleamine-containing neurons project to the forebrain and the spinal cord (Morgane and Stern, 1974; Baumgarten and Schlossberger, 1984; Conzolazione and Cuello, 1984). The dorsal raphe nucleus (DRN, B7) projects to the amygdaloid complex, hippocampal formation, lateral and posterior cortex, ventral thalamus and corpus striatum, the fibres overlapping with projections of the medial raphe nucleus (MRN, B8). More rostral fibers of the forebrain projections innervate the tuberculum olfactorium, septum, nucleus accumbens, transition cortex and main olfactory bulb. Both raphe nuclei appear to have minor descending projections to the brainstem and spinal cord (Baumgarten and Schlossberger, 1984).

Lesions of the MRN, but not the DRN, result in a spontaneous increase of adrenal TH (Quik et al., 1977; Sourkes, 1983) and favour the induction of adrenomedullary ornithine decarboxylase (EC 4.1.1.17) by dopaminergic agents (Almazán et al., 1982). Electrolytic lesions of the MRN and DRN are reported to produce decreases in forebrain serotonin of 20 and 50%, respectively (Quik et al., 1977). Because of the differential function of these two serotonin-rich nuclei in regard to adrenal enzyme induction, and because of the role of cerebral serotonin loss in the induction of adrenal DBH (Lima and Sourkes, 1985), it was now of interest to determine the relative roles of these two major raphe nuclei in the inducing action of reserpine. For this purpose we have made use of rats with electrolytic lesions of the brain.

3.I.3. Materials and Methods

Male Sprague-Dawley rats (290 ± 10 g) were purchased from Canadian Breeding Farms and Laboratories Ltd., St. Constant, Quebec. The animals were kept in individual cages under controlled lighting (12 h on/12 h

off) and fed ad libitum.

Animals submitted to surgery were anesthetized with 300 mg/kg ip of chloral hydrate (Fisher Scientific, Montreal, Quebec). Anodal electrolytic lesions were produced by passing 3 mA for 10 sec for DRN and 2 mA for 7 sec for MRN through a stainless steel monopolar electrode. Stereotaxic coordinates were taken from Paxinos and Watson (1982): for DRN, L 0, A 1.2 and V 6.5 mm and for MRN, L 0, A 1.2 and V 8.5 mm. Sham operated animals were treated as lesioned, except that no current was passed through the electrode. Five days later reserpine was given ip in a dose of 2.5 mg/kg daily for 3 days. At the termination of the experiments, the rats were deeply anesthetized with sodium methohexital, 65 mg/kg given ip,and the adrenals were removed and placed on ice. The glands were homogenized with a Teflon homogenizer in 1 ml of 0.05 M Tris buffer pH 7.4 containing 0.1% Triton X-100. Homogenates were centrifuged at 10,000 g for 10 min. The supernatant fraction was kept at -70°C until used for assay of DBH.

Adrenal DBH activity was determined according to Molinoff's procedure (Molinoff et al., 1971). Phenylethanolamine N-methyltransferase was purified from bovine adrenal medulla by the method of Díaz Borges (Díaz Borges et al., 1978), as specified previously (Lima and Sourkes, 1985).

To verify the site of the lesion the brains were immersed in formalin for 2-4 weeks, after which the brain stem was dissected. The parts for sectioning were placed in 30% sucrose for 24-48 h. Frozen sections of 50 um were prepared and stained with cresyl violet.

Each value is expressed as mean ± standard error (SE). Analysis of variance (ANOVA) was carried out to assess the significance of

differences (Winer, 1971).

3.I.4. Results

Representative lesions of MRN and DRN are shown in the Figure. Corresponding sections from the brain of sham-operated animals did not present any evidence of cell loss or histological change as a result of introducing the electrode.

Reserpine, given for 3 days, caused an increase of 77% in adrenal DBH activity in animals with a sham DRN operation (Table). Lesion of the DRN alone did not affect the resting level of adrenal DBH; but when reserpine was administered to rats with such a lesion, there was an increase of 69% in enzyme activity. This increase was not significantly different from that observed with reserpine in the sham-operated group (Table).

In rats sham-operated in the MRN region, reserpine produced an increase of 112% (Table). Lesioning of the MRN itself yielded a small, but not statistically significant, increase in adrenal DBH activity. However, when reserpine was administered to rats with lesions in this nucleus there was an increase of 197% in activity (Table). The effect of this lesion on the response to reserpine was statistically significant ($P < 0.025$; legend to Table). Thus, lesions of the MRN, but not the DRN, potentiated the inducing action of reserpine on adrenal DBH.

3.I.5. Discussion

Previous reports have shown that reserpine increases the activity of some adrenal enzymes (Thoenen et al., 1969; Molinoff et al., 1971; Ciaranello et al., 1975). This action with respect to DBH is potentiated by the systemic administration of PCPA or the icv injection of DHT (Lima and Sourkes, 1985), and is attenuated by the administration of serotonin

agonists (Lima and Sourkes, 1985). In contrast to this, although a significant decrease of central catecholamines is necessary for the induction of DBH to take place at all, additional measures to favour depletion of cerebral catecholamines, in conjunction with reserpine, do not potentiate the action of that drug (Lima and Sourkes, 1985). It has been previously observed that serotonergic neurons provide a negative input to the adrenal gland in regard to the regulation of the activity of TH (Quik et al., 1977; Sourkes, 1983) and ornithine decarboxylase (ODC) (Almazán et al., 1982) in that organ. These neurons seem to originate in the MRN (Quik et al., 1977). The present study shows that a lesion of the MRN, in contrast to a DRN lesion, potentiates the inducing action of reserpine on adrenal DBH activity. The injection of the neurotoxin DHT into the MRN (but not DRN) produces a similar result (Lima and Sourkes, 1985). These results indicate that serotonergic neurons of the MRN play a specific role in regulating the adrenal DBH content. Present evidence does not permit one to decide whether the MRN neurons involved in regulation of the three adrenal enzymes mentioned (TH, DBH, ODC) are the same or different.

The nervous connections of the MRN and DRN are apparently quite similar (Consolazione and Cuello, 1984; Baumgarten and Schlossberger, 1984). However, the role of the MRN in regard to neural induction of several adrenal enzymes, taken together with the failure of DRN to affect those inductions, makes it obvious that these nuclei do actually differ in their innervation. There is, in fact, documented evidence for such differences. For example, the MRN (but not the DRN) receives afferent fibres from the B9 area (Aghajanian and Wang, 1977), as well as other (noradrenergic) fibres from the A1/A2 region (Massari et al.,

1979). The DRN (but not the MRN) receives innervation from the nucleus tractus solitarii and the medial aspect of the preoptic nucleus; in fact there is evidence for an afferent connection from the MRN to the DRN (Aghajanian and Wang, 1977). As for efferent fibres, the major projection of the MRN is to the hippocampus and olfactory bulb, the DRN to the striatum Azmitia and Segal, 1978). However, these projections do not seem to be exclusive (Lorens et al., 1974; Kellar et al., 1977). It is, of course, possible that the connections between these two raphe nuclei and other regions, e.g. the locus coeruleus (Koslowski et al., 1974), are quite specific, without overlapping one another.

There is also neurochemical evidence of differential functions of these two nuclei. Intracisternal injection of neurotensin (Long et al., 1984) or parenteral administration of apomorphine (Lee and Geyer, 1982) to rats increases the serotonin content of the DRN, but not the MRN. McCulloch has observed that lesions of these nuclei modify glucose utilization of the brain, but the specific regions affected are different for MRN and DRN (McCulloch et al., 1984). In pharmacological experiments the catalepsy caused by administration of haloperidol to rats is attenuated by a lesion of the DRN, but there is no effect from damage to the MRN (Kozlowski et al., 1984).

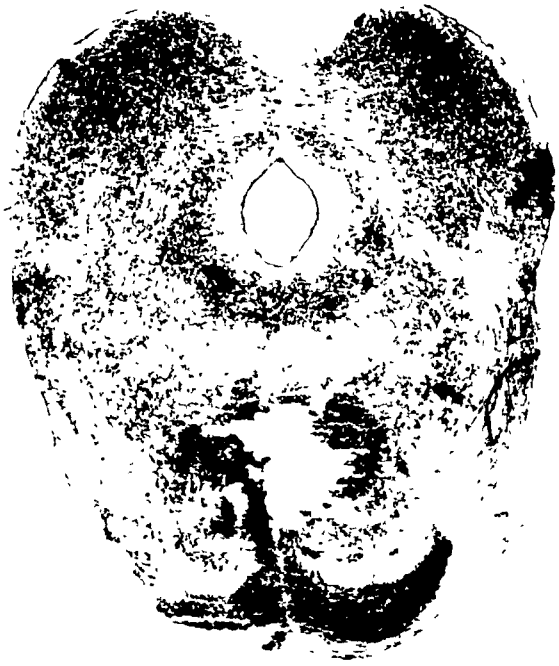
The present experiments thus contribute to the evidence of distinctive regulatory functions of these two raphe regions. Further experiments will be needed to determine which of the afferent and efferent connections of the MRN are important to the adrenomedullary regulatory function of that structure.

TABLE I. EFFECTS OF LESIONS IN THE RAPHE NUCLEI ON ADRENAL DBH ACTIVITY OF RESERPINIZED RATS.

	DBH activity (nmoles/30 min per adrenal)			
Treatment	N	DRN	N	MRN
Sham-operated	6	10.8 ± 0.7	9	8.1 ± 0.7
Sham-operated + reserpine	7	19.1 ± 3.4	11	17.2 ± 2.9
Lesioned	6	11.1 ± 1.9	9	11.4 ± 1.5
Lesioned + reserpine	9	18.3 ± 1.5	10	24.0 ± 1.2

Lesions were made as described in Materials and Methods, 5 d before the first of 3 daily injections of reserpine, given in a dose of 2.5 mg/kg ip. Rats were sacrificed 18 h after the last injection. In the DRN experiment ANOVA of 2 replicates was carried out with extraction of the sum of squares for experiments (1 degree of freedom), treatments (3), and interaction (3). Significance of differences between means was calculated from the mean square for remainder (10.7795, 20 degrees of freedom). Probabilities are as follows: effect of reserpine, $P < 0.001$; effect of reserpine in rats with DRN lesion, $P < 0.005$. In the MRN experiment ANOVA of 3 replicates was carried out with extraction of the sum of squares for replicates (2 df), treatments (3 df), and interaction (6 df). Significance of differences between means was calculated from the mean square for remainder (31.9985, 27 df). Probabilities are as follows: effect of reserpine in sham-operated rats, $P < 0.005$; effect of reserpine in rats with MRN lesion, $P < 0.001$; effect of MRN lesion on the action of reserpine, $P < 0.025$.

A



B

Figure 1

Representative lesions of
(A) medial raphe nucleus, and
(B) dorsal raphe nucleus,
in a frontal plane section through
the caudal midbrain

3.I.6. References

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3.J. Pharmacological analysis of the neurotransmitter mechanisms regulating phenylethanolamine N-methyltransferase and dopamine beta-hydroxylase in the adrenal gland.

3.J.1. Abstract

The ip administration of reserpine daily for 4 days to rats brings about an increase of adrenal phenylethanolamine N-methyltransferase (PNMT) activity. However, the combination of the systemic administration of p-chlorophenylalanine (PCPA) and reserpine for 3 days produces an earlier increase in this adrenal enzyme. The effect is significantly reduced in the denervated gland. Prior administration of 5,7-dihydroxytryptamine (DHT) icv to rats greatly potentiates the inducing effect of reserpine. On the other hand, the depletion of catecholamines by giving rats alpha-methyl-p-tyrosine (AMPT) ip or 6-hydroxydopamine (6OHDA) icv does not alter the action of reserpine on adrenal PNMT. PCPA, DHT, AMPT and 6OHDA do not have any effect by themselves on adrenal PNMT, but the combination of PCPA and AMPT each given ip causes increased adrenal PNMT activity. The administration of dopamine agonists, a treatment that increases adrenal TH, does not modify adrenal PNMT or DBH. We conclude that, just as in the case of DBH, the induction of PNMT by reserpine involves depletion of catecholamines and serotonin, the depletion of serotonin having the more powerful effect. A monoaminergic (serotonergic) inhibitory pathway is involved in the central regulation of adrenal PNMT activity.

3.J.2. Introduction

The activities of adrenal tyrosine hydroxylase (TH, EC 1.14.16.2), dopamine beta-hydroxylase (DBH, EC 1.14.2.1), and phenylethanolamine N-methyltransferase (PNMT, EC 2.1.1.28) are increased by stressors and by certain drug treatments (Ciaranello and Black, 1971; Kvetňanský et al., 1970; Kvetňanský, 1980; Thoenen, 1970). The response of adrenal TH to such treatments has been extensively studied in this laboratory and

elsewhere in order to elucidate the role of central dopaminergic (Quik and Sourkes, 1976), noradrenergic (Gagner et al., 1983), serotonergic (Quik and Sourkes, 1977), and cholinergic (Lewander et al., 1977) systems that regulate the increases. Less is known about neurotransmitters concerned with DBH activity of the adrenal gland of the rat, but cholinergic (Lewander et al., 1977) and monoaminergic fibres (Lima and Sourkes, 1985) are certainly involved. We now examine the regulation of the activity of a third adrenal enzyme involved in catecholamine synthesis, viz. PNMT. For these studies we have used rats given pharmacological agents that affect monoaminergic functions. In some experiments adrenal DBH was also measured for comparative purposes.

3.J.3. Materials and Methods

Male Sprague-Dawley rats (200 ± 10 g or 290 ± 10 g) were purchased from Canadian Breeding Farms and Laboratories Ltd., St. Constant, Quebec. The rats were kept in individual cages under controlled lighting (12 h on/12 h off) and were fed ad libitum.

Animals submitted to surgery were anesthetized with 300 mg/kg ip of chloral hydrate (Fisher, Scientific Co., Montreal, Quebec). Splanchnicotomy was performed in the left adrenal gland as previously described (Lima and Sourkes, 1985). The stereotaxic coordinates for the intracerebroventricular (icv) injection of neurotoxins were P 1.0, L 1.5 and V 3.5 mm. The ventricular target site was confirmed by injection of methylene blue. A Hamilton syringe with 26-gauge needle was used for the injections. 6-Hydroxydopamine hydrochloride (6OHDA) and 5,7-dihydroxytryptamine creatinine sulfate (DHT) (Sigma, St. Louis, MI) were injected icv in 20 µl of 0.1% ascorbic acid in saline. Doses of the neurotoxins are given as the weight of the salt. Reserpine (Sigma, St.

Louis, MI) was dissolved in a solution of 0.5% methylcellulose. Alpha-methyl-p-tyrosine hydrochloride (AMPT) and p-chlorophenylalanine hydrochloride (PCPA) were obtained from Sigma; apomorphine from F.E. Cornell and Co., Montreal, Quebec; piribedil from Laboratoires Servier, Neuilly-sur-Seine, France; and clonidine hydrochloride from Boehringer Ingelheim, Inc., Burlington, Ontario.

Rats were deeply anesthetized with Brietal (sodium methohexital), 65 mg/kg given intraperitoneally (ip), and then the adrenals were removed and placed on ice. The glands were homogenized with a Teflon homogenizer in 1 ml of 0.05 M Tris buffer pH 7.4, containing 0.1% Triton X-100 for DBH assay and in 1 ml of 0.05 M phosphate buffer pH 7.4, containing 0.15 KCl, 0.1 mM dithiothrietol and 1 mM EDTA. Homogenates were centrifuged at 10,000g for 10 min. The supernatant fraction was kept at -70° C for up to 4 days prior to assay.

Adrenal DBH was determined according to the method of Molinoff et al. (Molinoff et al., 1971). PNMT was purified from bovine adrenal medulla by the procedure of Díaz Borges (Díaz Borges et al., 1978) as specified previously (Lima and Sourkes, 1985). Adrenal PNMT was determined by the method of Yu (Yu, 1978).

Each value represents a mean ± standard error (SE). Adrenal DBH and PNMT activities are expressed as umoles and nmoles, respectively, of octopamine per 30 min per adrenal. Significance of the differences between means was calculated by Student's t-test. Analysis of variance (ANOVA) was done in indicated experiments (Winer, 1971).

3.J.4. Results

Action of reserpine on PNMT activity

It has been shown that three injections of reserpine given over a period of six days lead to about 20% increase of PNMT in the adrenal

gland (Ciaranello and Black, 1971). The experimental period could be effectively shortened by administration of this monoamine depletor to rats daily for 4 days, with an increase of 30% ($P < 0.025$) in the activity of adrenal PNMT (Table I, Expts. A and C). However, three daily injections were insufficient to induce the enzyme (Table I, Expt. B).

Serotonergic mechanism

The systemic administration of a single large dose of PCPA, an inhibitor of tryptophan hydroxylase (Koe and Weissman, 1966), to rats produces a small mean increase of adrenal PNMT activity (Table I, Expt. B), but this is not statistically significant. The injection of DHT icv, in order to produce central serotonergic denervation (Baumgarten et al., 1982), also did not affect adrenal PNMT activity significantly (Table I, Expt. C). However, when either of these treatments was combined with the administration of reserpine, there was potentiation of the effects of that drug (Table I, Expts. B and C).

PCPA in combination with reserpine given for four days was further tested in rats that had been hemisplanchnicotomized. For the intact adrenal, the effects on PNMT activity (Figure 1) were similar to those obtained previously. However, in the case of the denervated gland reserpine either alone or with PCPA no longer caused an increase of PNMT activity.

Catecholaminergic mechanism

Two treatments affecting catecholaminergic mechanisms were tested with respect to adrenal PNMT activity. 6OHDA, given by icv injection, in order to bring about central catecholaminergic denervation (Ungerstedt, 1968), had no significant effect on adrenal PNMT activity

nor did it alter the effect of reserpine given to rats for four days (Table I, Expt. A). The second treatment was the ip administration of AMPT, an inhibitor of TH that produces a great decrease in catecholamine levels in the central nervous system and the periphery (Spector, 1966). The adrenal PNMT activity of control rats was 3.78 ± 0.45 umoles per 30 min per adrenal (N=3) and that of AMPT-treated animals was 4.37 ± 0.25 (N=3) umoles per min per adrenal. The difference between these means (13%) was not statistically significant.

The effects of two dopamine agonists, piribedil and apomorphine, were also tested. These drugs, given ip for three days in dosage schedules that invariably result in large increases of adrenal TH (Quik and Sourkes, 1976; 1977) were tested in a four-day schedule. Neither one induced PNMT in the adrenal gland of the rat. Thus, it seems that the increase of adrenal PNMT does not involve dopaminergic regulation. Similarly, clonidine, tested at two dose levels (Gagner et al., 1983) did not modify adrenal PNMT activity (data not shown); this suggests that a noradrenergic mechanism is not implicated in the regulation of adrenal PNMT in the rat. Comparison experiments in which adrenal DBH was measured showed that this enzyme is also unaffected by the catecholamine agonists used (Table III).

Monoaminergic interaction

Because of the non-discriminating action of reserpine in releasing monoamine neurotransmitters, an experiment was carried out in which PCPA and AMPT were administered alone in order to test their depleting actions on serotonin and catecholamines, respectively (TableII). As before neither drug acting alone had an effect. However, the combination of these two increased adrenal PNMT by 44% above control value ($P < 0.01$). Thus, both types of monoamine, catecholamines and serotonin,

must undergo depletion in order to produce induction of PNMT in the adrenal gland of the rat.

3.J.5. Discussion

Reserpine is a very well known inducer of adrenal TH, DBH and PNMT, acting mainly through a neural mechanism (Zigmond and Bowers, 1981) but there are distinct differences in the forms of response of these enzymes. Thus, twenty-four hours after a single injection of reserpine there is already an increase in adrenal TH (Reis et al., 1975). However, two daily injections for two successive days are necessary to see the same effect on adrenal DBH activity (unpublished data) and a longer period of time, up to four days in the present study, is necessary to bring about an increase of adrenal PNMT. It is known that all three adrenal enzymes are under neural control, and that DBH and PNMT are additionally subject to humoral regulation (Ciaranello, 1980). The effect of humoral regulation is especially prominent in the case of PNMT. Thus, a longer period of stimulation is necessary to detect the neural effect on adrenal PNMT.

Although treatments (PCPA, ip; DHT, icv) that result in extensive depletion of cerebral serotonin in the raphe area and forebrain (Lima and Sourkes, 1985) bring about significant increases of adrenal TH activity (Quik and Sourkes, 1977), they do not influence adrenal PNMT (Table I) or DBH (Lima and Sourkes, 1985). However, such treatments given prior to reserpine potentiate the action of that inducer (Table I), just as was previously observed for adrenal DBH (Lima and Sourkes, 1985). The results with catecholamine depletion show that, just as in the case of serotonin-depleting agents, neither 6OHDA (Table I, Exp.A) nor AMPT (Table II) affects adrenal PNMT activity. In contrast todrugs

causing depletion of serotonin, 6OHDA did not influence the action of reserpine at all (Table I). These results emphasize the importance of specifically reducing the serotonergic component for induction of adrenal PNMT by reserpine through the neural route.

Comparison of the actions of DHT and PCPA with reserpine shows that the former was more effective under our experimental conditions. It is possible that the difference lies in the release by DHT (given icv) of additional modulators from serotonergic nerve endings. This actually occurs after central administration of DHT, in that there is depletion of substance P and thyrotropin releasing hormone, in parallel with serotonin, from the ventral spinal cord (Gilbert et al., 1982).

The experiments with individual monoamine-depleting agents show that cerebral loss of serotonin or catecholamines alone is not sufficient to induce adrenal PNMT. Earlier we found that catecholamine depletion does not increase adrenal TH (Quik and Sourkes, 1976) or DBH (Lima and Sourkes, 1985). However, the simultaneous decrease of serotonin and catecholamines, achieved by administering AMPT to rats that previously had received a single injection of PCPA, produced a significant increase in adrenal PNMT activity (Table II), just as in the case of DBH (Lima and Sourkes, 1985). These results indicate (i) that in order for reserpine to act as inducer of adrenal DBH and PNMT, depletion of both types of monoamine is necessary (Figure 1); and (ii) that this response is neurally mediated (Figure 1, Expt. C). Thus, a central serotonergic pathway is exerting a net inhibitory effect on the control of adrenal PNMT activity, just as has previously been recognized for TH (Quik and Sourkes, 1977) and DBH (Lima and Sourkes, 1985). However, it can not be concluded on the basis of the present evidence that the serotonergic tracts are the same in all three cases.

Present attempts to detect a dopaminergic mechanism in the regulation of adrenal DBH and PNMT were unsuccesful. Similarly the use of clonidine did not provide evidence for a noradrenergic mechanism in regard to either of these enzymes. The failure of catecholaminergic stimulation to affect adrenal DBH and PNMT contrasts with the importance of these mechanisms, especially the dopaminergic function, in the regulation of adrenal TH (Gagner et al., 1983; Quik and Sourkes, 1976) and, hence, points up the specificity and differential control of the central pathways involved in the regulation of catecholamine-synthesizing enzymes in the adrenal gland.

Table I. Effect of PCPA, DHT, 6OHDA and reserpine on adrenal PNMT activity

Experiment	Treatment	N	PNMT activity umoles per 30 min per adrenal Without reserpine	N	With reserpine
A	Control	5	6.16 ± 0.34	7	8.00 ± 0.52
	6OHDA	5	7.53 ± 0.55	5	8.70 ± 0.62
B	Control	5	6.67 ± 0.39	5	6.57 ± 0.38
	PCPA	5	7.79 ± 0.39	5	9.36 ± 0.67*
C	Control	3	3.79 ± 0.55	4	4.99 ± 0.23**
	DHT	3	4.46 ± 0.36	5	7.61 ± 0.58***

Each value is Mean ± SE. Experiment A: 6OHDA was given icv in a dose of 200 μg in 20 μl of 0.1% ascorbic acid in saline 4 d before the first injection of reserpine. Experiment B: PCPA was administered in a dose of 300 mg/kg once 24 h before the first of three daily injections of reserpine. Experiment C: DHT was administered icv, 175 μg in 20 μl of 0.1% ascorbic acid in saline 30 min after 20 mg/kg of desimipramine and 4 d prior to the first of 4 daily ip injections of reserpine. Reserpine was given in a dose of 2.5 mg/kg and the animals were killed 24 h after the last injection.

* $P < 0.005$ with respect to control with or without reserpine and $P < 0.05$ with respect to PCPA without reserpine.

** $P < 0.025$ with respect to control without reserpine.

*** $P < 0.01$ with respect to DHT without reserpine and control with reserpine.

Table II. Effect of PCPA and AMPT on adrenal PNMT activity

Treatment	N	PNMT activity μmoles per 30 min per adrenal
Control	4	5.45 ± 0.32
PCPA	3	4.59 ± 0.70
AMPT	3	4.91 ± 0.17
Both	4	7.83 ± 0.62

Each value is Mean ± SE. PCPA was administered in a dose of 300 mg/kg ip once 48 h before the first injection of AMPT, whic was given in a dose of 200 mg/kg per day in two injections for 4 d. Animals were sacrificed 18 h after the last injection. ANOVA was carried out with extraction of sum of squares for AMPT (1 degree of freedom), for PCPA (1), interaction (1) and remainder (7). Significance of the difference between means was calculated from the mean square for remainder (0.7302). Probabilities are as follows: control vs. both < 0.01; AMPT vs. both < 0.005; PCPA vs. both < 0.05.

Table III. Effect of piribedil, apomorphine and clonidine on adrenal PNMT and DBH activities

Experiment	Treatment	PNMT N	PNMT μmoles per 30 min per adrenal	DBH N	DBH nmoles per 30 min per adrenal
A	Control	11	5.05 ± 0.50	3	5.05 ± 0.54
	Piribedil	11	5.96 ± 0.93	6	6.45 ± 0.82
B	Control	6	5.34 ± 0.19	6	6.37 ± 0.33
	Apomorphine	6	5.41 ± 0.51	5	7.03 ± 0.91
C	Control	4	7.33 ± 0.37	4	7.90 ± 1.08
	Apomorphine	6	7.44 ± 0.45	6	7.19 ± 0.49
D	Control	6	5.34 ± 0.19	6	6.37 ± 0.33
	Clonidine	6	5.93 ± 0.40	6	6.48 ± 0.81
E	Control	4	7.33 ± 0.37	4	7.90 ± 1.08
	Clonidine	6	7.29 ± 0.12	6	7.13 ± 1.73

Each value is Mean ± SE. Experiment A: Piribedil was given in a dose of 50 mg/kg ip twice a day for 4 d. Experiment B: Apomorphine was given in a dose of 3 mg/kg 4 tiems a day for 4 d. Experiment C: Apomorphine was given in a dose of 10 mg/kg 4 times a day for 4 d. Experiment D: Clonidine was given ip in a dose of 2 mg/kg twice a day for 4 d. Experiment E: Clonidine was given in a dose of 15 μg/kg twice a day for 4 d. Animals were killed 18 h after the last injection.

Figure 1. Effect of hemisplanchnicotomy, reserpine and PCPA on adrenal DBH activity. Hemisplanchnicotmy was carried out 5 d before starting the treatment. PCPA was given in a single dose of 300 mg/kg ip 2 d before first injection of reserpine. Reserpine was given daily in a dose of 2.5 mg/kg sc for 4 d. Sacrifice was done 24 h after last injection. ANOVA was carried out with extraction of sum of squares for splanchnicotomy (1 degree of freedom), treatments (3), interaction (3) and remainder (17). Significance of differences between means was calculated from the mean square for remainder (0.3138). Probabilities are as follows:

Difference between	Intact	Denervated
Control vs. reserpine	< 0.05	NS
Reserpine vs. PCPA	< 0.05	NS
Both vs. reserpine	< 0.025	= 0.05
Both vs. PCPA	< 0.005	NS
Both vs. control	< 0.005	NS

Reserpine intact vs. reserpine denervated side	< 0.01
Both intact vs. both denervated side	< 0.005

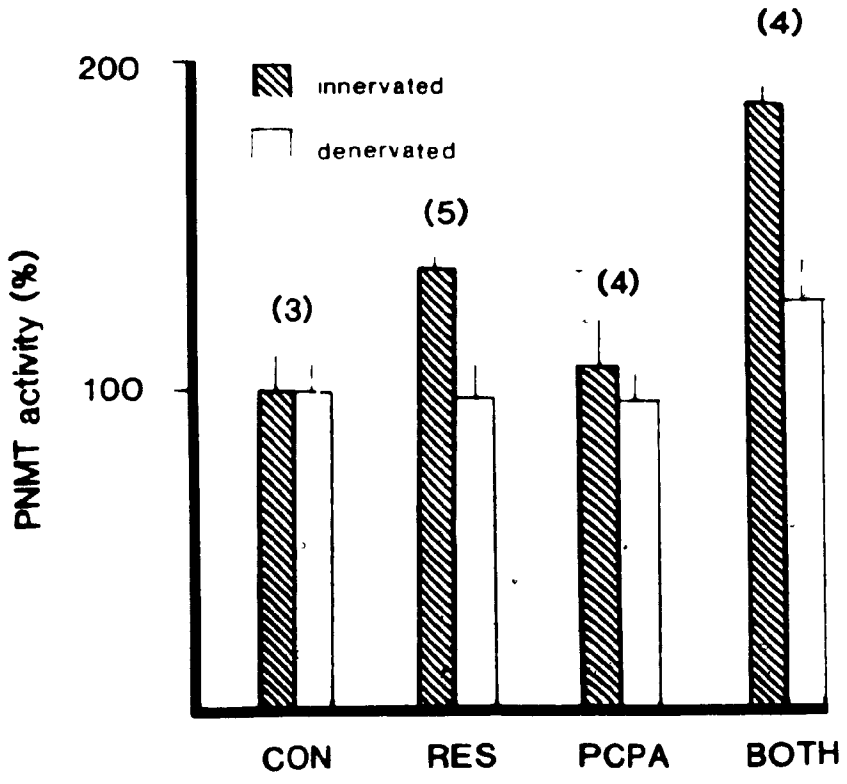
200
100
PNMT activity (%)
innervated
denervated
(3)
(5)
(4)
(4)
CON
RES
PCPA
BOTH

Figure 1

3.J.6. References

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3.K. Cholinergic and gabaergic regulation of dopamine beta-hydroxylase activity in the adrenal gland of the rat

3.K.1. Abstract

The administration of oxotremorine together with methylatropine to rats produces a dose-dependent increase of adrenal dopamine beta-hydroxylase (DBH) activity. This effect is abolished by denervation of the gland and by cycloheximide. The Km for tyramine is not affected by the transsynaptic induction of DBH by oxotremorine. The induction is selective, because similar treatment does not affect adrenal dopa decarboxylase or lactate dehydrogenase in the adrenal gland. The combination of 6-hydroxydopamine icv or propranolol ip does not alter the effect of oxotremorine on adrenal DBH. However, propranolol reduces the tremorigenic action of the muscarinic agonist. The systemic administration of p-chlorophenylalanine or the icv injection of 5,7-dihydroxytryptamine prior to oxotremorine treatment does not affect the increase of adrenal DBH. Progabide, a $GABA_A$- and $GABA_B$-receptor agonist that effectively crosses the blood brain barrier, reduces the effect of oxotremorine in a dose dependent manner. Muscimol, given by either of two routes -icv at a constant rate (Alzet minipump) or ip-, produces significant decreases of adrenal DBH activity and attenuates the action of oxotremorine. Denervation of the gland abolishes the effect of muscimol ip in decreasing adrenal DBH activity. Baclofen, a $GABA_B$-receptor agonist, has no effect by itself nor does it affect the action of oxotremorine. None of these GABA agonists has any *in vitro* effect on adrenal DBH activity. Bicuculline, a $GABA_A$-receptor antagonist, reverses the action of progabide in oxotremorine-treated rats with respect to adrenal DBH activity, partially blocks the effect of muscimol, and enhances the increase obtained with oxotremorine. Thus, GABA seems to be involved in the regulation of adrenal DBH activity, and at least some of its effect in this regard is mediated by interaction

with a central cholinergic system. In contrast to this, the results of these experiments provide no basis for roles for central or peripheral catecholamines and serotonin in interaction with the central action of oxotremorine on this enzyme.

3.K.2. Introduction

The muscarinic agonist oxotremorine (Cho et al., 1962) effects the induction of dopamine beta-hydroxylase (DBH, EC 1.14.2.1) and tyrosine hydroxylase (TH, EC 1.14.16.2) in the rat adrenal gland by central neural action (Lewander et al., 1977), but neither the site at which it acts in the nervous system nor the efferent pathway is known. Central cholinergic fibres interact with various neurotransmitters and some of these relationships have been documented. Thus, oxotremorine decreases the turnover rate of acetylcholine in many regions of the brain (Nordberg, 1978), but it accelerates the turnover rate of cerebral serotonin (Haubrich and Reid, 1972) and elevates the plasma concentrations of epinephrine and norepinephrine in conscious rats (Weinstock et al., 1978). Moreover, catecholaminergic centres influence the action of cholinergic neurons (Ladinsky et al., 1980; Weinstock et al., 1978). The inhibitory neurotransmitter gamma-aminobutyric acid (GABA) blocks the activity of cholinergic neurons in the striatum and elsewhere (Scatton and Bartholini, 1979, 1980), and GABA agonists, such as progabide and muscimol, diminish the rate of turnover of cerebral acetylcholine (Scatton and Bartholini, 1980, 1982). Because of such interactions that affect turnover and levels of central neurotransmitters it was considered worthwhile to extend the investigation of the induction of rat adrenal DBH by oxotremorine, this time with attention to the effects of drugs able to mimic or modify

actions of some of the neurotransmitters mentioned on that induction of that enzyme.

3.K.3. Materials and Methods

Male Sprague-Dawley rats (200 or 290 ± 10 g) were purchased from Canadian Breeding Farms and Laboratories Ltd., St. Constant, Quebec. The animals were kept in individual cages under controlled lighting (12 h on/12 h off) and fed ad libitum.

Animals submitted to surgery were anesthetized with 300 mg/kg ip of chloral hydrate (Fisher Scientific, Montreal, Quebec). Hemisplanchnicotomy was performed and verified as described previously (Lima and Sourkes, 1985). The stereotaxic coordinates for the intracerebroventricular (icv) injection of the neurotoxins were P 1.0, L 1.5 and V 3.5 mm (Paxinos and Watson, 1982). A craniotomy was performed at the site corresponding to the injection. The ventricular target site was confirmed by injection of methylene blue. A Hamilton syringe with 26-gauge needle was used for the injections. For the continuous microinfusion of drugs, osmotic minipumps (Model 2001, Alzet Corporation), were used. An L-shaped stainless steel cannula was prepared from 21-gauge tubing. The tip of the cannula had a length of 3.5 mm; in order to guarantee its depth, a small stop was made with epoxy resin. The longer part of the L was connected to 8 cm polyethylene tubing, internal diameter 0.76 mm, the other end of which was attached to the mini-osmotic pump flow moderator. The mini-pumps were filled according to the procedure described in the manufacturer's instruction manual. The L-shaped cannula-catheter was also filled with the solution to be delivered.

6-Hydroxydopamine (6OHDA), 200 µg and 5,7 dihydroxytryptamine (DHT), 175 µg (Sigma, St. Louis, MI), were injected by the

intracerebroventricular (icv) route in 20 µl of 0.1% ascorbic acid in saline. Injection time was one minute in order to avoid asymmetric uptake (Baumgarten et al., 1982). The drugs used (dose, route of administration and source) were: DL-propranolol, 50 mg/kg intraperitoneal (ip); muscimol, 0.5 and 3 mg/kg ip or 50 ng/day icv; p-chlorophenylalanine (PCPA), 300 mg/kg ip (Sigma, St. Louis, MI); and baclofen, 10 mg/kg ip (Ciba-Geigy, Montreal, Quebec). These were dissolved in saline. Progabide 100 and 50 mg/kg ip (Synthelabo, Paris), and gamma-vinylGABA, 1g/kg (Merrell International, Strasbourg, France), were prepared in 0.5% methylcellulose. Oxotremorine (Aldrich Chemicals, Milwaukee, WI) was given sc in a dose of 0.5 mg/kg 30 min after atropine methyl bromide, 5 mg/kg ip (Sigma, St. Louis, MI).

Rats were deeply anesthetized with sodium methohexital, 65 mg/kg and then the adrenal glands were removed and placed on ice. The glands were homogenized with a Teflon homogenizer in 1 ml of 0.05 M Tris buffer pH 7.4 containing 0.1% Triton X-100. Homogenates were centrifuged at 10,000g for 10 min. The supernatant fraction was kept at -70°C for up to 4 days prior to assay.

Fragments of adrenal gland were pre-incubated in 2 ml of Krebs-Ringer-bicarbonate, containing 0.4% glucose for 10 min, and then incubated for 30 min in the presence of 23 µM progabide, 4 µM muscimol, 7 µM baclofen or 580 µM gamma-vinylGABA, respectively.

Adrenal DBH activity was determined according to the procedure of Molinoff et al. (Molinoff et al., 1971). PNMT was purified from bovine adrenal medulla by the method of Diaz Borges et al. (Díaz Borges et al., 1978), as specified previously (Lima and Sourkes, 1985).

Each value is expressed as a mean ± standard error (SE).

Significance of the difference between mean was calculated by Student t-test. Analysis of variance (ANOVA) was done in indicated experiments (Winer, 1971).

3.K.4. Results

Preliminary experiments served to verify the inductive effect of oxotremorine on adrenal DBH. Activity of the enzyme increased in a dose-dependent fashion with daily administration of 0.1, 0.25 or 0.5 mg of oxotremorine per kg body weight for four days, the increase being statistically significant ($P < 0.05$) only with the last dose. Tremor was observed at each of these dose levels. Further experiments confirmed the finding that denervation of the adrenal abolishes this induction (cf. Lewander et al., 1977). The induction of DBH by oxotremorine was blocked by 1 mg/kg per day of cycloheximide (unpublished data, this laboratory). Kinetic studies of dialyzed DBH preparations from adrenals of oxotremorine-treated and control rats showed that the Km for tyramine as substrate was similar in the two cases, 0.09 mM and 0.11 mM, respectively. In order to asses the target selectivity of the induction adrenal lactate dehydrogenase and aromatic amino acid decarboxylase activities were measured after administering the drug to rats in a dose that induces TH, DBH (Lewander et al., 1977) and ornithine decarboxylase (Ramírez-González et al., 1980). These two enzymes were unaffected by this treatment (Lima and Sourkes, unpublished data).

Catecholaminergic mechanisms: Several reports suggest that cholinergic inputs via muscarinic receptors can influence the activity of catecholaminergic systems (Morgan and Pfeil, 1979; Korczyn and Eshel, 1979; Ladinsky et al. 1976, 1980). In order to reduce the efficacy of central catecholaminergic fibres that might be involved in the inductive

effect of oxotremorine two types of experiment were carried out. In the first, the neurotoxin 6OHDA (Breese and Traylor, 1970; 1971) was injected into the cerebral ventricles of rats. 6OHDA was without significant effect on adrenal DBH activity, either by itself or in combination with oxotremorine (Table I). Because oxotremorine causes the release of adrenal catecholamines (Weinstock et al., 1979) and because the beta-adrenergic agonist propranolol can reduce the tremor provoked by the drug (Weinstock et al., 1978), the second experiment involved giving propranolol ip to rats in a dose of 5 mg/kg before each injection of oxotremorine. The blocker reduced the tremorigenic effect of oxotremorine slightly but did not influence the effect of the drug on adrenal DBH (Table II). Moreover, it had no significant effect of its own on enzymic activity.

Serotonergic mechanisms: Two experiments were conducted for the purpose of evaluating a possible serotonergic-cholinergic interaction with respect to adrenal DBH induction. Such interaction has been described for some biological functions (Haubrich and Reid, 1972; Robinson 1982, 1984). As shown in Table III the administration of DHT icv in order to destroy the endings of forebrain serotonergic neurons (Baumgarten et al., 1982) did not affect the adrenal DBH activity when given alone or in combination with oxotremorine. In the second experiment PCPA (Koe and Weissman, 1966) was injected in a schedule that diminishes sharply the cerebral serotonin content through inhibition of tryptophan hydroxylase. As in the case of the neurotoxin, this inhibitor had no significant effect on adrenal DBH activity either by itself or with oxotremorine.

GABAergic mechanisms: Progabide, a GABA agonist (Worms et al.

1982), was administered to rats in three similar experiments, the dose of the drug in the second and third experiments being 50% greater than in the first. Results obtained in the latter two experiments are presented in Table IV, this shows the interaction with oxotremorine was given at a time when the maximal action of progabide could be expected to occur (Worms et al. 1982). Analysis of the data reveals that oxotremorine increased adrenal DBH activity by 67%. However, when this drug was given with progabide the increase was only 28%, the reduction in effect being statistically significant (see footnote, Table IV). With the higher dose of progabide there was a decrease of 24% in the effect of oxotremorine ($P < 0.025$) (Figure 4); with the lower dose of the drug the decrease was 17% ($P > 0.05$) (data not shown). Thus, a reduction of the inducing effect of oxotremorine was produced by the conjoint administration of the GABA agonist, progabide.

In order to study further the action of progabide on the induction of adrenal DBH by oxotremorine bicuculline, a $GABA_A$ receptor blocker (Curtis et al., 1971), was administered to rats receiving either oxotremorine or the combination of oxotremorine and progabide. Bicuculline appeared to favour the action of oxotremorine, in increasing the induction of DBH with that drug by 21% ($P < 0.05$). Moreover, in rats in which the inducing effect of oxotremorine had been abolished with progabide administration, as in Table 4, the bicuculline now counteracted that effect ($P < 0.01$, Table V).

Other GABA agonists, such as muscimol (Brehm et al. 1972), baclofen (Fox et al. 1978) and gamma-vinylGABA (Jung et al. 1977), each having a distinct mechanism of action, were also tested. Muscimol, in a dose of 0.5 mg/kg ip did not produce any effect on adrenal DBH activity or on the action of oxotremorine on this enzyme (data not shown). A higher

dose of the GABA agonist (3 mg/kg) produced a decrease of adrenal DBH activity (26%), without affecting the net central effect of oxotremorine (Table VI). In addition, when muscimol was given icv at a constant rate of 50 ng/day, a great decrease of adrenal DBH activity was observed (53%), a result that an otherwise effective dose of oxotremorine could not completely overcome (Table VI). Nevertheless, the relative increase in adrenal DBH activity elicited by oxotremorine is essentially unaffected by concomitant administration of muscimol (Table VI).

The potent effect of muscimol by itself in decreasing rat adrenal DBH activity over a 3-day period (Table VI) raised the question of whether this effect is exerted neurally or otherwise. To study this rats were hemisplanchnicotomized and then given muscimol ip. The results in Table VII demonstrate clearly that the DBH-reducing action of muscimol is exerted on the innervated adrenal ($P < 0.05$) but not on the denervated gland ($P > 0.05$).

Baclofen did not alter either the effect of oxotremorine or the resting activity of adrenal DBH (Table VIII). Gamma-vinylGABA produced a decrease of 25% in adrenal DBH activity, but this change was not statistically significant; moreover, gamma-vinylGABA had no significant effect on the increase observed when oxotremorine was given to the animals (Table VIII).

Fragments of adrenal gland were incubated in the presence of corresponding concentrations of the GABA agonists. None of these drugs tested in vivo modified DBH activity in vitro (data not shown).

3.K.5. Discussion

Lewander et al. (1977) demonstrated that the cholinergic agonist, oxotremorine, induces TH and DBH in rat adrenals. The induction of

these enzymes by oxotremorine persists even if the animals are given methylatropine, an antagonist that has very little access to the brain after peripheral administration (Witter et al., 1973). Immunochemical studies have shown that there is an actual increase in the amount of TH protein produced in this way (Lewander et al., 1977). This also seems to be the case for adrenal DBH, because the injection of cycloheximide into rats blocks the increase observed after they are given oxotremorine (unpublished data). There is a selectivity to the induction: in addition to DBH, TH (Lewander et al., 1977) and ornithine decarboxylase (Ramírez-González et al., 1980) of the adrenal are induced, but lactate dehydrogenase and aromatic amino acid decarboxylase activities are unaffected by treatment of rats with oxotremorine (Lima and Sourkes, unpublished data).

The abolition of the induction of DBH by denervation of the gland (Lewander et al., 1977) is amply verified by the data of Table I. Thus, we are dealing with a central cholinergic mechanism that affects the regulation of adrenal DBH. Some other adrenomedullary enzymes are inducible through stressors acting by way of central monoaminergic pathway (Sourkes, 1985). Thus, the induction of TH is influenced by dopaminergic, serotonergic (Quik and Sourkes, 1977) and noradrenergic (Gagner et al., 1983) systems; DBH and PNMT by monoaminergic, but excluding at least dopaminergic pathways (Lima and Sourkes, 1985); and ornithine decarboxylase through dopaminergic, serotonergic (Almazán et al., 1983) and cholinergic neurons (Ramírez et al., 1980). The present work has aimed at determining whether any of these types of neuron interact with cholinergic fibres in the regulation of adrenal DBH.

The efficacy of central catecholaminergic fibres was reduced in rats by giving them an intracerebroventricular injection of 6OHDA, a

neurotoxin that reaches many of the dopamine- and norepinephrine-containing endings in the brain and causes them to degenerate and lose their stores of amines. By itself this did not affect the activity of adrenal DBH (Table II), nor did it affect the induction by oxotremorine. The beta-adrenergic blocker propranolol was also tested. Although it is known to abolish the tremor caused by oxotremorine (Weinstock et al., 1978), it did not modify the inducing effect of that cholinergic drug on adrenal DBH (Table II). This demonstrates that tremor can be separated from other effects of central muscarinic stimulation. Moreover, propranolol had no effect by itself on rat adrenal DBH.

To test the possible involvement of central serotonergic fibres rats received serotonin depletors, namely, PCPA to inhibit tryptophan hydroxylase, and DHT, delivered to the ventricular space of the brain in order to exert its neurotoxic effect on serotonergic nerve endings. Neither substance significantly affected adrenal DBH activity by itself or in combination with oxotremorine (Table III). This is in sharp contrast to the potentiating effect of these agents, given in combination with reserpine, on the induction of adrenal DBH (Lima and Sourkes, 1985). The fact that a serotonergic mechanism is involved in the induction by reserpine but not by oxotremorine illustrates the specificity of the central influences regulating such peripheral functions. The results also demonstrate that the existence of such an inhibitory control (serotonergic) does not necessarily cause a decreased response of adrenal medullary enzymes when the animal is under stress, for other pathways are available to stimulate some of those adrenomedullary functions. Thus, the organism is assured of plasticity in this regulation.

Cerebral cholinergic systems are known to interact with GABA-containing neurons. For example, GABA prevents secretion of corticotropin releasing factor by incubated hypothalamic fragments when acetylcholine is applied to them, and picrotoxin in turn blocks this effect of GABA (Jones and Hillhouse, 1977). The analgesic response to GABA-receptor activation is blocked by atropine (Andree et al., 1983). Bicuculline increases acetylcholine efflux from the cerebral cortex (Gardner and Webster, 1973). It is in the light of such findings that GABA agonists were tested in this work. The first of these was progabide a $GABA_A$ and $GABA_B$ agonist, a synthetic substance that penetrates the blood-brain barrier rapidly so that its level in brain may rise to 1.5-2 times the plasma concentration (Lloyd and Morselli, 1982). Its metabolites are also GABA agonists with long half-lives (Lloyd and Morselli, 1982). The present results show that progabide significantly decreases the effect of oxotremorine on adrenal DBH activity by 24% in a dose of 150 mg/kg daily; when given in a dose of 100 mg/kg it results in non-significant decrease of 17% (see text above and Table IV). In unpublished experiments we have found that progabide has no effect on the induction of adrenal DBH by reserpine. The effect of progabide seems to be mediated by $GABA_A$ receptors, because bicuculline blocks the action of the GABA agonists on the induction of adrenal DBH by oxotremorine (Table V).

Other GABA agonists, such as muscimol, gamma-vinylGABA, and baclofen were also tested. Muscimol is a highly active $GABA_A$ agonist, but penetrates the blood-brain barrier only to a very small extent (Maggi and Enna, 1979; Enna and Gallager, 1983), so that high doses or icv injections must be used to obtain a central effect. This drug decreases the resting activity of adrenal DBH (Tables VI and VII), but

does not significantly impair the action of oxotremorine. The result with muscimol given ip could be explained as stemming from the stimulation of $GABA_A$ receptors in the adrenal medulla, with a decrease in the release of catecholamines (Kataoka et al., 1984); the decrease would produce a feedback inhibition of adrenal DBH. In this case, a central action could not be expected because muscimol penetrates very poorly into the brain. However, the icv administration of muscimol in a small constant dose produces a great decrease in adrenal DBH activity (Table VI), and this suggests that muscimol does have a central action. An increase of 16% in DBH activity is produced by oxotremorine in rats that are also receiving muscimol, compared with the 21% increase in the animals receiving oxotremorine alone, even though basal level of enzyme activities has been drastically lowered by the muscimol. These results support the possibility of a central inhibitory pathway that involves GABA as neurotransmitter and affects adrenal function. A general inhibitory effect of GABA on tonic stimulatory pathways maintaining resting levels of adrenal DBH could also occur. The GABA actions are probably mediated by way of A-receptors because of the effect of muscimol on adrenal DBH activity (Tables VI and VII), and also because of the lack of effect of baclofen, a $GABA_B$ agonist (Curtis et al., 1974; Fox et al., 1978; Hill and Bowery, 1981) on this activity (in the presence or absence of oxotremorine) (Table VIII). This view is strengthened by the fact that bicuculline, a specific blocker of $GABA_A$-receptors (Enna, 1983), blocks the action of progabide on the inducing effect of oxotremorine.

The administration of high doses of gamma-vinylGABA, a GABA-T inhibitor, increases GABA content in the brain (Jung et al., 1977).

Treatment of rats with this drug decreases adrenal DBH by 25% and the action of oxotremorine on this enzyme by 16%, but these effects are not statistically significant (Table VIII). Perhaps higher doses of the inhibitor would increase the endogenous GABA sufficiently to inhibit some of the adrenal functions, but we did not exceed a dose of 1 g/kg.

We can conclude that, whereas central catecholaminergic or serotonergic systems do not seem to interact with the central cholinergic system involved in the induction of adrenal DBH in the rat, a central inhibitory action of GABA reduces the activity of this enzyme and can impair the effect of oxotremorine upon its activity.

TABLE I. **Effect of denervation on the increase of adrenal DBH activity in oxotremorine treated rats**

Treatment	Adrenal DBH activity nmoles per 30 min per adrenal			
	N	Innervated	N	Denervated
Sham operated	3	8.0 ± 0.5	3	8.7 ± 0.6
Sham operated + oxotremorine	3	15.5 ± 0.7*	3	13.7 ± 0.6*
Splanchnicotomized	4	9.1 ± 0.7	4	9.6 ± 0.7
Splanchnicotomized + oxotremorine	4	13.7 ± 1.1**	4	8.1 ± 0.3

Each values is Mean ± SE. Left splanchnicotomy was done 4 days before the first injection of the drug. Oxotremorine was administered sc in a dose of 0.5 mg/kg and 30 min after 5 mg/kg ip of methylatropine twice a day, separated by 5 h interval for 4 days. Animals were killed 18 h after the last injection.

* $P < 0.005$ with respect to sham operated

** $P < 0.025$ with respect to splachnicotomized.

TABLE II. Effect of oxotremorine and 6OHDA on adrenal DBH activity

Expt	Treatment	N	DBH activity nmoles per 30 min per adrenal: Without oxotremorine	N	With oxotremorine
1	Control	5	13.1 ± 0.9	7	28.9 ± 3.9*
	6OHDA	3	15.1 ± 2.4	7	25.1 ± 1.1*
2	Control	5	8.7 ± 0.7	5	13.8 ± 0.7*
	Propranolol	5	9.4 ± 0.9	6	15.3 ± 0.7*

Each value is Mean ± SE. 6OHDA, 200 µg icv in 20 µl of 0.1% ascorbic acid in saline 4 days before the first injection of oxotremorine. Propranolol was given ip in a dose of 5 mg/kg 40 min before each of 2 daily injections of oxotremorine. Oxotremorine was administered sc in a dose of 0.5 mg/kg 30 after 5 mg/kg of methylatropine twice a day for four days. Animals were killed 18 h after the last injection

* $P < 0.025$ with respect to control.

TABLE III. **Effect of PCPA, DHT and oxotremorine on adrenal DBH activity**

Expt.	Treatment	N	DBH activity nmoles per 30 min per adrenal Without oxotremorine	N	With oxotremorine
1	Control	4	9.3 ± 0.8	4	15.4 ± 1.4*
	DHT	4	10.5 ± 1.9	7	13.9 ± 0.9*
2	Control	5	11.1 ± 0.5	5	23.3 ± 2.3*
	PCPA	5	14.8 ± 1.7	4	26.8 ± 3.1*

Each value is Mean ± SE. PCPA, 300 mg/kg ip and DHT, 175 µg icv in 20 µl 0.1% ascorbic acid in saline were given 24 h and 4 d before the first injection of oxotremorine respectively. Animals were sacrifice 18 h after last injection.

* $P < 0.01$ with respect to the corresponding without oxotremorine.

TABLE IV. **Effect of progabide and oxotremorine on adrenal DBH activity**

Treatment	N	DBH activity nmoles per 30 min per adrenal Without oxotremorine	N	With oxotremorine
Control	9	7.5 ± 0.7	9	12.5 ± 0.8
Progabide	7	7.4 ± 0.7	9	9.5 ± 0.8

Each value is Mean ± SE. Progabide, 100 mg/kg ip at 9.00 p.m. and 50 mg/kg at 2:00 p.m. Oxotremorine, 0.5 mg/kg sc 30 min after 5 mg/kg ip of methylatropine was given at 11·00 a.m. and 4·00 p.m. for 3 d. Animals were killed 18 h after the last injection. ANOVA of the two experiments was carried out with extraction of sum of squares for experiments (1 degree of freedom), treatments (2), interaction (3) and remainder (26). Mean square of remainder (3.6253). Probabilities are: control vs. oxotremorine < 0.001, progabide vs. both < 0.05 and oxotremorine vs. both < 0.005.

TABLE V. **Effect of oxotremorine, progabide and bicuculline on adrenal DBH activity**

Treatment	N	DBH activity nmoles per 30 min per adrenal
Oxotremorine	7	11.8 ± 0.6
Oxotremorine + progabide	7	9.3 ± 0.6
Oxotremorine + bicuculline	8	14.3 ± 0.8
Oxotremorine + progabide + bicuculline	8	12.4 ± 0.9

Each value is Mean ± standard error. Two daily injections of each drug were performed: Bicuculline, 1 mg/kg ip at 10:00 a.m. and 1:00 p.m. Progabide, 150 mg/kg ip per day at 10:15 a.m. and 1:15 p.m. Oxotremorine, 0.5 mg/kg sc 30 min before methylatropine, 5 mg/kg ip, at 12:00 m. and 3:00 p.m. for 3 days. Probabilities are as follows: oxotremorine vs oxotremorine + progabide < 0.05; oxotremorine vs oxotremorine + bicuculline < 0.05; oxotremorine + progabide vs oxotremorine + progabide + bicuculline < 0.01. Bicuculline given alone was not effective.

TABLE VI. **Effect of muscimol and oxotremorine on adrenal DBH activity**

Treatment	DBH activity nmoles per 30 min per adrenal			
	N	A	N	B
Control	5	10.6 ± 0.4	4	13.8 ± 0.7
Oxotremorine	5	16.1 ± 0.9	3	16.7 ± 0.4
Muscimol	5	7.8 ± 0.5	5	6.5 ± 0.8
Both	6	12.3 ± 1.1	4	7.6 ± 0.9

Each value is Mean ± SE. In A, muscimol was given ip in a dose of 3 mg/kg one h before each injection of oxotremorine. In B, muscimol was administered icv into the right lateral ventricle in a dose of 50 ng per day by an osmotic mini-pump at a rate of 1 µl per h, starting 18-24 h before the first injection of oxotremorine and during the treatment. Oxotremorine was given in a dose of 0.5 mg/kg sc twice a day for 3 d and 30 min after 5 mg/kg ip of methylatropine. Rats were sacrifice 18 h after the last injection. ANOVA was carried out in each experiment with extraction of sum of squares: Muscimol ip: treatments (1 degree of freedom), interaction (1) and remainder (14). Significance of the differences between means was calculated from the mean square for remainder (3.5798). Probabilities are as follows: control vs. oxotremorine $P < 0.005$, control vs. muscimol $P < 0.05$, oxotremorine vs. both $P < 0.01$ and muscimol vs. both $P < 0.005$. Muscimol icv: treatments (1), interaction (1) and remainder (12). Mean square for remainder (2.4622). Control vs oxotremorine, $P < 0.05$, control vs muscimol, $P < 0.001$, oxotremorine vs both $P < 0.001$ and control vs both, $P < 0.005$.

TABLE VII. **Effect of muscimol on adrenal DBH activity of hemisplanchnicotomized rats**

Treatment	N	DBH activity nmoles per 30 min per adrenal	
		Intact adrenal	Denervated adrenal
Control	3	7.6 ± 0.3	8.3 ± 0.8
Muscimol	5	6.3 ± 0.4	9.1 ± 0.9

Each value is mean ± standard error. Hemisplanchnicotomy was performed 5 days before the first injection of the drug. Muscimol was given ip in a dose of 3 mg/kg twice a day for 3 days. Probabilities calculated from one tailed t test show: control vs muscimol (intact adrenal) < 0.05; muscimol (intact adrenal) vs muscimol (denervated adrenal) < 0.025.

TABLE VIII. **Effect of baclofen, gamma-vinyl GABA and oxotremorine on adrenal DBH activity**

Treatment	N	DBH activity nmoles per 30 min per adrenal: Without oxotremorine	N	With oxotremorine
Control	5	8.9 ± 0.8	5	13.4 ± 1.2
Gamma-vinyl GABA	3	6.7 ± 0.4	3	11.2 ± 0.1
Control	5	6.8 ± 0.3	5	11.7 ± 1.1
Baclofen	5	6.8 ± 0.7	6	10.0 ± 0.9

Each value is Mean ± SE. Gamma-vinyl GABA was given in a dose of 1000 mg/kg ip 2 h before each injection of oxotremorine. Baclofen was given in a dose of 10 mg/kg 1 h before each injection of oxotremorine. Oxotremorine was administered sc twice a day in a dose of 0.5 mg/kg 30 min after 5 mg/kg ip of methylatropine for 3 d. Animals were killed 18 h after the last injection.

3.K.6. References

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3.L. Effect of corticotropin-releasing factor on adrenal DBH and PNMT activities and plasma corticosterone levels.

3.L.1. Introduction

Corticotropin releasing factor (CRF) has been characterized as a 41-aminoacid residue-containing polypeptide with potent adrenocorticotropin (ACTH) and beta-endorphin releasing activity (Vale et al., 1981). Systemic administration of antiserum against CRF or a CRF antagonist blocks these effects (Rivier et al., 1982; 1984). CRF is anatomically distributed in many brain regions, including some outside the areas related to the regulation of anterior pituitary function (Swanson et al., 1983). CRF given icv to rats and dogs yields a dose dependent elevation of plasma noradrenaline and adrenaline concentrations (Brown and Fisher, 1984), and produces hyperglycemia (Brown et al., 1982) Central administration of CRF also affects cardiovascular functions (Fisher et al., 1982), Thus, CRF not only acts by releasing several hormones, but also could be the first mediator of the stress response. For these reasons, the central action of CRF on adrenal DBH and PNMT regulation was tested.

3.L.2. Materials and Methods

Animals. Male Sprague-Dawley rats (290 ± 10 g) were obtained from Canadian Breeding Farms and Laboratory Ltd., St. Constant, Quebec. The animals were kept in individual cages under controlled lighting (12 h on/12 h off) and were fed ad libitum.

Animals submitted to surgery were anesthetized by the combination of ketamine, 60 mg/kg, and xylazine, 10 mg/kg, im (Harkness, 1980). Hemisplanchnicotomy was carried out and verified as previously described (Lima and Sourkes, 1985). The stereotaxic coordinates for the intracerebroventricular (icv) administration of the peptide were P 1.0, L 1.5 and V 3.5 mm (Paxinos and Watson, 1982). The ventricular target

site was confirmed by injection of methylene blue. Alzet osmotic minipump were used for the central administration of ovine CRF (Peninsula, Belmont, CA, U.S.A.) by constant infusion, according to the procedure previously described (Lima and Sourkes, 1985). The CRF was administered in a dose of 100 ng/day in saline. Control animals received the vehicle in the same way.

Reserpine (Sigma Chemicals, St. Louis, U.S.A.) was administered sc in a dose of 2.5 mg/kg daily for three days.

Six to seven days after the placement of the cannula and the infusion the animals were deeply anesthetized with sodium methohexital, 65 mg/kg ip and adrenals were removed and placed on ice. Each gland was homogenized with a Teflon homogenizer in 1 ml of 0.05 M Tris buffer pH 7.4, containing 0.1% Triton X-100 for DBH determination; and in 1 ml of 0.05 M phosphate buffer pH 7.4, containing 0.15 M KCl, 0.1 mM dithiothreitol, and 1 mM EDTA for PNMT determination. Homogenates were centrifuged at 10,000 g for 10 min, an the supernatant fraction was stored at -70°C until used in enzyme assay. Adrenal DBH was determined by the method of Molinoff et al. (Molinoff et al., 1971), with some minor modifications. Final volumes were reduced to one-tenth of that specified in the Molinoff procedure, except for the partially purified bovine PNMT, used in the second step of the assay; this was reduced to one-fifth the specified volume.

Bovine adrenal PNMT was purified from bovine adrenal medulla by the procedure of Diaz Borges et al. (Díaz Borges et al., 1978) as previously described (Lima and Sourkes, 1985). Adrenal PNMT was determined by the micromethod of Yu (Yu, 1978).

Plasma corticosterone was determined by a competitive binding radioassay (Murphy et al., 1963; Murphy and Wagner, 1972).

Each value represents a mean ± standard error (SE). Significance of the difference between means was calculated by Student's t test. Analysis of variance (ANOVA) was done in indicated experiments (Winer, 1971).

3.L.3. Results

CRF was administered by the way of osmotic minipumps as described in Materials and Methods. A dose of 100 ng/day of this hormone increased adrenal DBH and PNMT activities without affecting plasma corticosterone significantly (Table I). As in previous studies (Lima and Sourkes, 1985), reserpine given for three days caused increases of both DBH and PNMT in the adrenals of rats. However, reserpine administered to rats receiving CRF icv caused no significant further modification in the activity of the enzymes under study (Table I). Plasma corticosterone was elevated similarly by reserpine and by the combination of reserpine and CRF.

CRF was also given icv to hemisplanchnicotomized rats. Both DBH and PNMT increased in activity in the innervated adrenal as before (Table II). Denervation of the gland did not alter significantly the activity of these enzymes (Table II). CRF caused increases of DBH and PNMT activity in the denervated gland, but only the latter increase was statistical significant.

CRF given sc increases plasma corticosterone, because the pituitary gland, outside of the blood brain barrier, is accessible to the action of the peptide (Veldhius and De Wied, 1984). CRF was administered for 3 days in two daily doses (Table 3). Adrenal DBH and PNMT, and plasma corticosterone were determined. CRF sc increased PNMT activity by 40%, but adrenal DBH was not significantly modified under these conditions.

3.L.4. Discussion

The role of CRF as the principal physiological stimulator of ACTH release (Vale et al., 1981; Plotsky and Vale, 1984) is based on the fact that systemic administration of either antisera against CRF or CRF receptor-antagonists (Rivier et al., 1982; 1984) prevents ACTH secretion. CRF is anatomically distributed in hypothalamic and extrahypothalamic nuclei. Several limbic nuclei, such as the lateral amygdaloid nucleus, periventricular thalamic nuclei and bed nucleus of the stria terminalis contain CRF (Palkovits et al., 1985). Thus, in addition to its hormonal functions as releaser of ACTH and beta-endorphins, CRF has CNS actions, and could be the first mediator of the stress response. In fact, CRF acts within the brain to stimulate sympathetic outflow (Brown et al., 1984), and in this way affects cardiovascular functions (Brown et al., 1984) and glucose metabolism (Fisher et al., 1982). Also, the icv administration of CRF results in behavioural changes (Britton et al., 1982; Morley and Levine, 1982; Sutton et al., 1982). Veldhuis and De Wied (Veldhuis and De Wied, 1984) reported in the rat that one hour after a single icv injection of CRF in doses of less than 300 ng, central effects could be observed, without increase in plasma corticosterone. They also show that the sc injection of 300 ng of CRF produces an increase in plasma corticosterone corresponding to the increase observed in the icv injected rats. The administration of CRF, one µg/h, by an osmotic minipump connected to the jugular vein produces a decrease in plasma luteinizing hormone (LH) levels up to the 7th day, suggesting stability of the compound in such conditions (Rivier and Vale, 1984).

We administered CRF icv by means of the osmotic minipumps, in a dose of 100 ng/day, a dose that did not significantly increase plasma

corticosterone levels (Table 1). However, both DBH and PNMT activities increased significantly with this treatment. Denervation of the gland blocked the elevation of DBH but not PNMT activity, suggesting that the activation of the adrenal medulla by CRF has two components: neural and humoral. These effects are evident in the case of doubly regulated enzymes, such as DBH and PNMT (Ciaranello, 1980).

There is considerable evidence that adrenal PNMT is regulated by the pituitary-adrenal axis (Wurtman and Axelrod, 1966; Ciaranello et al., 1975), to the extent that glucocorticoids or ACTH administration do not significantly elevate adrenal PNMT activity in normal animals, but prevent the decrease observed in hypophysectomized rats (Ciaranello, 1978). In the present experiments CRF given sc was able to increase adrenal PNMT activity in the intact animal. Probably CRF administered by the sc route is potent enough to elevate corticosteroids in the adrenal portal system of the rat, these steroids cause increases of PNMT activity (Wurtman and Axelrod, 1966). CRF might have a local effect in the adrenal medulla favouring an increase of PNMT.

CRF given sc, as indicated in Table 3, did not significantly increase adrenal DBH activity.

Our results are preliminary, but nevertheless suggest that the activation of the adrenal medulla by CRF has two components: neural and humoral. Peripheral effects of the polypeptide need also to be studied.

Table I

Effect of CRF and reserpine on adrenal DBH and PNMT activities and plasma corticosterone.

Treatment	N	DBH	PNMT	Plasma corticosterone
Control	4	6.2 ± 0.3	3.9 ± 0.3	1.2 ± 0.2
Reserpine	5	9.5 ± 0.7[a]	5.2 ± 0.4[a]	1.9 ± 0.2[a]
CRF	5	9.3 ± 0.9[b]	5.5 ± 0.4[b]	1.7 ± 0.5
Both	5	10.1 ± 0.7[c]	6.0 ± 0.2[c]	2.3 ± 0.3[c]

Each value is mean ± SE. CRF was administered icv with an osmotic minipump in a dose of 100 ng/day for 6-7 days. Reserpine was given the 3 last days of the CRF administration in a dose of 2.5 mg/kg sc daily. Animals were killed 18 h after the last injection.

Significance with respect to corresponding control is:

a $P < 0.05$

b $P < 0.025$

c $P < 0.001$

Table II

Effect of CRF on adrenal DBH and PNMT activities and plasma corticosterone of hemisplanchnicotomized rats.

Enzyme	Treatment	N	Enzyme activity		▽Plasma corticosterone
			Intact adrenal	Denervated adrenal	
DBH	Control	3	14.8 ± 0.6	14.3 ± 1.8	1.8 ± 0.1
	CRF	4	20.6 ± 1.2*	18.8 ± 1.5	1.7 ± 0.2
PNMT	Control	3	2.9 ± 0.3	2.1 ± 0.3	1.7 ± 0.4
	CRF	4	3.6 ± 0.2*	3.2 ± 0.04*	1.8 ± 0.1

Each value is mean ± SE. Hemisplanchnicotomy was carried out 5 d before CRF, icv by an osmotic minipump in a dose of 100 ng/d for 7 days. Animals were killed on the 8th day. DBH, nmoles per 30 min per adrenal; PNMT, umoles per 30 min per adrenal; corticosterone, µg/ml.

* $P < 0.05$

Table III

Effect of CRF given sc on adrenal DBH and PNMT activities

Treatment	N	Enzyme activity DBH	PNMT
Control	6	13.9 ± 0.5	1.1 ± 0.1
CRF (Low dose)	6	15.3 ± 2.2	1.6 ± 0.1*
CRF (High dose)	6	15.7 ± 2.0	1.5 ± 0.1*

Each value is mean ± SE. CRF was given sc twice a day for 3 d. Low dose: 60 ng/d. High dose: 600 ng/d. DBH, nmoles per 30 min per adrenal; PNMT, umoles per 30 min per adrenal; corticosterone, µg/ml.

* $P < 0.05$

3.L.5. References

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4.- GENERAL DISCUSSION

The experimental work described in this thesis has been directed toward elucidating neural and other mechanisms involved in the regulation of two adrenomedullary enzymes DBH and PNMT. This has meant, in the first instance, attempting to discover which of the commonly accepted neurotransmitters play roles in the neural induction of these two enzymes. Reserpine, a drug that causes the temporary and reversible loss of monoamine neurotransmitters from the brain and other parts of the nervous system, has been used for two reasons: (1) it causes induction of DBH and PNMT in the adrenal glands; and (2) it lowers brain catecholamines and serotonin, among other substances in the brain, to low levels. It has hitherto been assumed that the second factor explains the first, but the relationship has not been clarified. In this work, the effect of reserpine in inducing adrenal DBH is significantly reduced by denervation of the gland. Nevertheless, a small increase is still observed, and this suggests (a) that the drug exerts a local effect by reducing adrenal catecholamines, or (b) that it acts through a humoral component as well. However, reserpine still increases the activity of this enzyme in hypophysectomized rats. Hence, the increase of adrenal DBH in reserpine-treated rats must be considered as mainly neurally mediated.

In order to analyze further the effects of the non-specific action of reserpine in decreasing brain amine concentrations, the administration of specific depletors of either catecholamines or serotonin was carried out in the experimental animals. It was established that these depleting agents, given individually, do not alter adrenal DBH activity, but the combination of a serotonin depletor and a catecholamine depletor, a treatment designed to mimic the action

of reserpine, increases enzyme activity. This result indicates that the induction is produced through the central depletion of both types of monoamine. The contribution of catecholamine depletion to the effect was confirmed, at least in the case of dopamine, by finding that the administration of dopaminergic agonists, well documented inducers of TH, does not alter adrenal DBH or PNMT in our experimental conditions. Thus, for TH induction dopamine sensitive receptors rostral to the thoracic spinal cord, as demonstrated by others, are important, but for DBH or PNMT induction such receptors have no role or need to be blocked e.g. by depletion of the presynaptic nerve endings.

It was found that serotonin agonists, but not catecholamine agonists, potentiate the effect of reserpine on adrenal DBH and PNMT; this suggests that the depletion of serotonin has a more powerful effect. In fact, the existence of a central serotonergic pathway with a net inhibitory action has previously been described for the regulation of adrenal TH. This pathway is also involved in the regulation of the other two inducible enzymes of catecholamine biosynthesis. The production of electrolytic lesions or the administration of small amounts of serotonin neurotoxins into the medial raphe nucleus, but not into the dorsal raphe nucleus, potentiates the effect of reserpine without significantly affecting the resting level of adrenal DBH. Thus, the serotonergic pathway to the adrenal medulla concerns TH, DBH and PNMT, and it has its centre in the medial raphe nucleus.

Cholinergic pathways were examined next. For this purpose oxotremorine, a muscarinic agonist, was used because of its known effect in inducing adrenal TH and DBH by a neural mechanism. The combined administration of oxotremorine and either catecholaminergic or serotonergic antagonists given peripherally or centrally does not

modify the effect of oxotremorine on adrenal DBH. These results suggest that the central cholinergic pathway involved in the regulation of adrenal DBH does not interact with catecholaminergic or serotonergic systems. Thus, the organism has available either a monoaminergic pathway (as elucidated in the experiments with reserpine) or a cholinergic mechanism, so that if one system were inactive for some reason, the other could be maintaining resting levels of the enzyme. This represents a plasticity in the central regulation of peripheral functions.

The administration of GABA-receptor agonists such as progabide and muscimol significantly reduces the increase of adrenal DBH in oxotremorine-treated rats. Because this effect of GABA-agonists is blocked by $GABA_A$-receptor antagonists it is suggested that this type of receptor is involved in the interaction with the central cholinergic system. Moreover, muscimol, a potent $GABA_A$-receptor agonist, decreases adrenal DBH, an effect that it is blocked by adrenal denervation. The action of muscimol could be due to the activation of a central inhibitory pathway to the adrenal gland or to a general inhibitory action of the neurotransmitter.

Some experiments were carried out with peptides, particularly corticotropin-releasing factor. This substance was infused into the cerebrospinal fluid by slow release from an Alzet pump. It increased adrenal DBH and PNMT activities without increasing plasma corticosterone. This finding supports a central action of the peptide independent of the stimulation of the hypothalamic-pituitary-adrenal axis. However, the effect of CRF on adrenal DBH activity is not completely abolished in denervated adrenals, and not at all affected in

the case of PNMT. This means that CRF has a dual effect: i) a neural action which is direct, or else mediated through adrenocorticotropin and beta-endorphins acting on some other neural substrate with connections to the adrenal medulla; and ii) a humoral effect mediated through the activation of anterior pituitary causing release of adrenocorticotropin and beta-endorphins and, thereby, adrenal activation.

5.- ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

1. Though reserpine is a well known inducer of adrenal enzymes, it was not hitherto clear where its action is exerted in this regard. Through a "pharmacological-biochemical dissection" of the drug action this work provides a clarification of the central action of reserpine. This was carried out by the central and peripheral administration of catecholamine and serotonin antagonists, given in combination to mimic the effect of reserpine.

2. This work supports the existence of a central serotonergic pathway with a net inhibitory action in the regulation of the adrenal enzymes, DBH and PNMT. Such a pathway has been previously described for the regulation of adrenal TH activity. Through the use of classical techniques it was demonstrated that this pathway has its centre in the medial raphe nucleus.

3. A central cholinergic system has been recognized as playing a role in the induction of the adrenal enzymes, TH and DBH, for about 10 years. This has now been investigated for possible interactions with other central systems. Thus, some GABA agonists decrease and the $GABA_A$ antagonist, bicuculline, enhances the effect of the muscarinic agent, oxotremorine, on adrenal DBH activity. Impairment of catecholaminergic or serotonergic functions does not affect oxotremorine action. This work provides the first evidence for the existence of a possible central GABA-cholinergic interaction concerning the regulation of adrenal DBH. The cholinergically induced increase of the synthesis of adrenal DBH is specific, for other adrenal enzymes such as dopa decarboxylase and lactate dehydrogenase do not increase with oxotremorine treatment.

4. Muscimol, given peripherally or icv by osmotic minipumps decreases adrenal DBH. The central inhibitory effect of muscimol on an adrenal enzyme is novel and suggests either a general inhibitory effect of the GABA agonist or an inhibitory pathway to the adrenal gland.

5. CRF has been suggested as a first mediator in the stress response. Another contribution of this work is that centrally administered CRF, given by means of an osmotic minipump in a dose small enough not to affect plasma corticosterone, increases adrenal DBH and PNMT activities. This result suggests that CRF has a central action independent of its stimulatory effect on the pituitary gland. The dual role of CRF, together with the dual regulation of adrenal DBH and PNMT, offers an interesting model for study of the control of adrenal functions.