NEUROHUMORAL REGULATION OF ADRENAL ORNITHINE DECARBOXYLASE ACTIVITY

by

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To the memory of my father, Eloy

Ph.D. Biochemistry

Guillermina Almazán

ABSTRACT

Neurohumoral Regulation of Adrenal Ornithine Decarboxylase

The aim of this study has been to elucidate the neural pathways involved in the regulation of adrenal ornithine decarboxylase (ODC) activity. Administration of the dopamine-receptor agonists apomorphine (APM) and piribedil (PBD) to rats led to an increase in ODC activity of both the adrenal medulla and cortex. These effects were blocked by giving the animals the dopaminergic antagonist haloperidol. The APM-induced increase in adrenomedullary ODC activity was largely prevented by denervation of the adrenal, transection of the spinal cord, and transection of the mesencephalon-diencephalon. Section of ventral spinal roots reduced the induction to varying extents, depending on the number of roots cut and their location between T_4 and T_{12}. The inducing effect of APM on adrenocortical ODC was abolished by hypophysectomy. Splanchnicotomy, rhizotomy and bilateral adrenal demedullation each attenuated the action of the drug. In contrast to this, section of the spinal cord or surgical isolation of the hypothalamus (preparation of "hypothalamic island") potentiated its effect. Impairment of serotonergic nerve function by systemic administration of p-chlorophenylalanine and intraventricular injection of 5,6'-dihydroxytryptamine or electrolytic lesions

potentiated the effect of APM in the adrenal medulla, but reduced it in the cortex. These observations suggest that adrenal ODC activity is predominantly regulated by one or more central facilitatory dopaminergic pathways. The pathway for the regulation of the medullary enzyme involves nuclei in the diencephalon-telencephalon and ultimately acts through the sympathetic nervous system. The pathway for the cortex involves the hypothalamus and acts via the anterior pituitary gland. These pathways include serotonergic components, which have opposite net effects on the induction of ODC produced by APM: inhibitory for the medulla and facilitatory for the cortex.

Ph.D. Biochimie

Guillermina Almazán

ABSTRACT

Régulation Neurohumorale de l'Ornithine Décarboxylase Surrénalienne

Cette étude avait pour but de déterminer les voies nerveuses impliquées dans la régulation de l'activité de l'enzyme ornithine décarboxylase (ODC) surrénalienne. L'injection d'apomorphine (APM) ou de piribédil (PBD), agonistes de la dopamine, a causé une augmentation de l'activité de l'ODC à la fois dans les tissus médullaires et corticaux chez le rat. Ces effets étaient bloqués en injectant l'animal avec de l'halopéridol, un antagoniste de la dopamine. L'augmentation de l'activité de l'ODC induite par l'APM dans les tissus médullaires fut en majeure partie éliminée par splanchnicotomie, une section transversale de la moëlle épinière ou une section transversale du mésencéphalo-diencéphale. Une section des racines ventrales de la moëlle a réduit l'induction à divers degrés, dépendant du nombre et de la localisation des racines ventrales entre les niveaux T_4 et T_{12}. L'effet inducteur de l'APM sur l'activité de l'ODC du tissu cortical fut aboli par une hypophysectomie. Une splanchnicotomie, une rhizotomie ou une démédullation bilatérale des surrénales ont toutes attenué l'action de la drogue. Cependant, une section transversale de la moëlle, ou l'isolement chirurgical de l'hypothalamus (préparation d'un "îlot hypothalamique"), ont amplifié l'effet de la drogue. Une altération du système sérotoninergique par injection périfique de para-chlorophénylalanine, par injection

intraventriculaire de 5,6-dihydroxytryptamine, ou par lésions électrolytiques ont amplifié l'effet inducteur de l'APM dans le tissu médullaire, mais ont réduit cet effet dans le tissu cortical. Ces observations suggèrent que l'activité de l'ODC surrénalienne est principalement contrôlée par une ou plusieurs voies dopaminergiques facilitatrices située au niveau du système nerveux central. La voie responsable pour le contrôle de l'enzyme médullaire implique des noyaux du diencéphale-telencéphale et opère via le système nerveux sympathique. La voie pour le tissu cortical implique l'hypothalamus et opère via l'hypophyse antérieure. Ces voies comprennent des composantes sérotoninergiques qui exercent des effets globaux opposés sur l'induction de l'ODC produite par l'APM: effet inhibiteur pour l'enzyme médullaire et facilitateur pour l'enzyme cortical.

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Almazan G, Ramirez-Gonzalez MD, Sourkes TL (1982) Effect of apomorphine, piribedil and haloperidol on adrenal ornithine decarboxylase activity of the rat. Neuropharmacology, in press (Chapter II of the thesis, except for the kinetic analysis and effect of cycloheximide).

Almazan G, Pacheco P, Vassilieff VS, Sourkes TL (1982) Adrenomedullary ornithine decarboxylase activity: its use in biochemical mapping of the origins of the splanchnic nerve in the rat. Brain Res, in press (Chapter IV).

Almazan G, Pacheco P, Sourkes TL (1982) Central dopaminergic regulation of adrenomedullary ornithine decarboxylase activity. Submitted (Chapter V).

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M.D. Ramirez-Gonzalez taught me the enzymatic assay and helped me to organize the experimental protocol of the first experiments.

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V.S. Vassilieff participated in the surgery for intraventricular injection of 5,6-dihydroxytryptamine and 6-hydroxydopamine and in a few experiments for the section of the spinal roots together with P. Pacheco and myself. She also homogenized the samples in some of the mentioned experiments.

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ABBREVIATIONS

ACTH	adrenocorticotropin
APM	apomorphine
ATP	adenosine triphosphate
cAPM	adenosine-3',5'-monophosphate
CNS	central nervous system
CRF	corticotropin releasing factor
CYC	cycloheximide
D	dorsal
d	day
DA	dopamine
DHT	5 ,6 -dihydroxytryptamine
DRN	dorsal raphe nucleus
E	epinephrine
EDTA	disodium ethylene diamine tetraacetate
h	hour
HLP	haloperidol
HPLC	high performance liquid chromatography
5HT	5-hydroxytryptamine; serotonin
L	lumbar
i.p.	intraperitoneal
µCi	microcurie
mCi	millicurie

MBH	mediobasal hypothalamus
min	minute
MRN	medial raphe nucleus
NE	norepinephrine
NGF	nerve growth factor
ODC	ornithine decarboxylase
6-OHDA	6-hydroxydopamine
P	probability
PBD	piribedil
PCPA	p-chlorophenylalanine
PC12	clonal cell line from rat adrenal pheochromocytoma
PLP	pyridoxal 5'-phosphate
pmol	picomole
PPO	2,5-diphenyloxazole
RNA	ribonucleic acid
rRNA	ribosomal RNA
SAM	S-adenosyl-L-methionine
SAMD	S-adenosyl-L-methionine decarboxylase
SAPN	sympathoadrenal preganglionic neurons
s.c.	subcutaneous
SEM	standard error of the mean
T	thoracic
V	ventral

CHAPTER I

Introduction

Ornithine decarboxylase (L-ornithine carboxylyase,EC 4.1.1.17; ODC) has been implicated in growth processes in a wide range of tissues and cultured cells. Regulation of ODC has received increasing attention because this enzyme acts at the first, and rate-limiting, step in the synthesis of polyamines (231).

The polyamines spermidine and spermine and their immediate precursor putrescine occur ubiquitously in living tissues, but are found in highest concentrations in tissues that actively synthesize protein and have a high RNA content (346). The physiological role of the polyamines is not entirely understood. However, numerous lines of evidence indicate that they play an essential role in the regulation of various cell functions, including DNA synthesis, transcription and translation, and in the modulation of membrane function and activity of numerous enzymes (348,156, 38).

The elucidation of the physiological factors involved in control of the biosynthesis of the polyamines would contribute to determining the biological role of these compounds. In this work, we further investigate the mechanism of regulation of ornithine decarboxylase in the adrenal gland of the rat.

A. POLYAMINES AND THE ROLE OF ORNITHINE DECARBOXYLASE IN THEIR BIOSYNTHESIS

The polyamines spermidine and spermine and the diamine putrescine (1,4-diaminobutane) are simple aliphatic amines. As indicated by their trivial chemical names, the polyamines were first detected in seminal fluid;

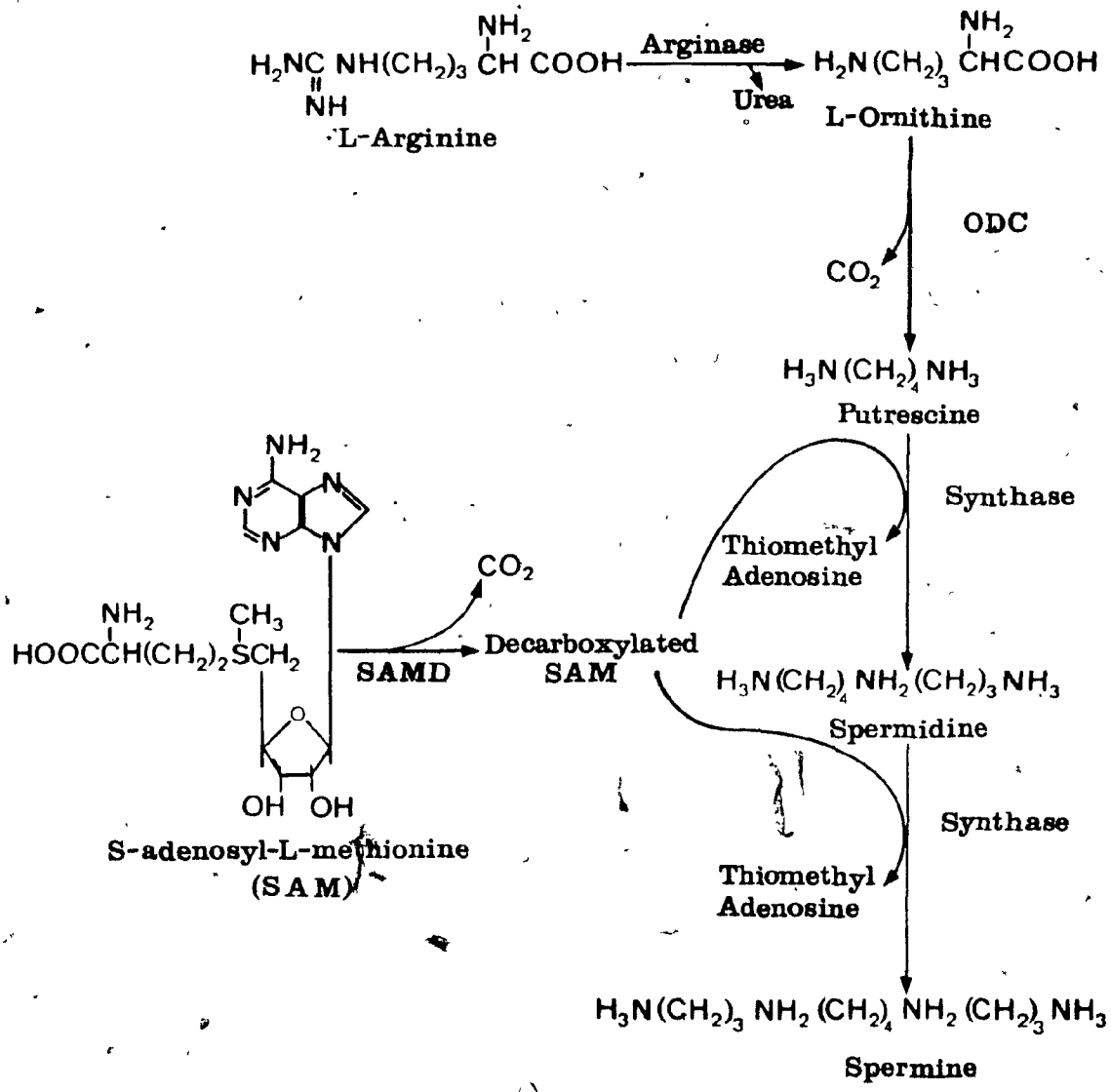


FIGURE 1

Biosynthetic pathway for the polyamines

and they are secreted by the prostate gland, the mammalian tissue with the highest concentration of these substances (346). Spermidine and spermine are present in significant concentrations (millimolar), whereas putrescine is usually present in much lower (nanomolar) amounts (370). However, putrescine accumulates in tissues stimulated to increase in size or cell number (304).

Jänne et al. (157) were the first to study the content of polyamines in different rat tissues. They showed that those tissues with the highest metabolic activity tended to have the highest concentration of spermidine and a relatively high spermidine/spermine ratio; and that the concentrations of both compounds decrease with age.

(a) Biosynthesis of polyamines

The primary precursors of polyamines are L-ornithine and L-methionine. The former is converted to putrescine by decarboxylation. L-Methionine is first activated with the aid of ATP to form S-adenosyl-L-methionine; this, then, is used as the donor of the propylamine moiety for the synthesis of higher polyamines.

Raina et al. (286) first showed that in the rat, within a few hours after partial hepatectomy, synthesis of spermidine from ^{14}C-methionine in the regenerating liver is markedly increased. They obtained similar results with ornithine, but not with putrescine (153,151) as the endogenous putrescine pool was enlarged many times in the regenerating liver.

The mechanisms of enzymatic synthesis of spermidine and spermine in the rat prostate gland were later elucidated by the work of Pegg and Williams-Ashman (266). Synthesis of polyamines *in vitro* was achieved with

preparations of regenerating rat liver and prostate (151,266).

Four enzymes are involved in polyamine biosynthesis: two decarboxylases and two synthases. They are all localized in the soluble fractions of homogenates of animal tissues (284,337,345,266). Putrescine is formed following decarboxylation of ornithine by ODC. Its basal activity is the lowest of the enzymesengaged in the polyamine synthesis (287), and it thus can be considered as the rate-controlling enzyme. ODC is a typical cytosol decarboxylase requiring pyridoxal phosphate as cofactor. Its striking inducibility (231) and its very short half-life (307) make it a unique representative among mammalian enzymes. Inhibition of ODC by putrescine, spermidine and spermine and other related amines has been observed in a number of physiological systems as well as in a variety of cell cultures. This enzyme will be fully described and its mechanism of regulation analyzed in the second part of this introduction.

A second decarboxylase, S-adenosyl-L-methionine decarboxylase (EC 4.1.1.50, SAMD), is required for the synthesis of spermidine. This enzyme has been extensively purified (263,264). It is the only mammalian decarboxylase to require pyruvate as cofactor (263,264,48). Its half-life is about 35-60 min (308,64), but its inducibility is less pronounced and less prevalent in mammalian organisms than in the case of ODC (213,285). SAMD is specifically stimulated by putrescine and related amines (266). This is effected partly by lowering the K_m for the substrate and partly by protecting the enzyme against inactivation (263,250). In some systems spermidine is a potent inhibitor of SAMD (141). Maudsley (213) has recently

suggested that after ODC has been stimulated and putrescine levels are increased SAMD becomes the rate-limiting enzyme in the synthesis of spermidine and spermine. The work of Oka and Perry (249) on the mammary gland; and the metabolic studies of Antrup and Seiler (7) in mouse brain provide support for the above hypothesis.

The two propylamine transferases, spermidine and spermine synthases, differ from one another. They have been partly purified from rat ventral prostate, liver and brain (267,159,116). These enzymes are turned over more slowly than the decarboxylases.

In addition to the three roles of putrescine in the synthesis of polyamines already mentioned (substrate for spermidine synthesis; inhibitor of ODC; and allosteric activator of SAMD), this diamine acts as a competitive inhibitor of spermine synthesis (265). Spermidine can also inhibit its own formation from putrescine and decarboxylated S-adenosyl-L-methionine (265).

(b) Turnover and degradation of polyamines

The turnover of endogenously synthesized polyamines has been studied in rat liver after single intraperitoneal doses of trace amounts of putrescine (310,305,238), ornithine (310,305), spermidine and spermine (334). Because, in normal liver, only negligible amounts of polyamines are synthesized from ^{14}C-ornithine, most of the studies have been performed in regenerating rat liver, where ODC activity is increased many fold. Spermidine turnover in regenerating rat liver has a half-life of 4-5 days, putrescine, about 120 min. On the other hand, newly synthesized spermine from the radioactive precursors declines little or not at all in the

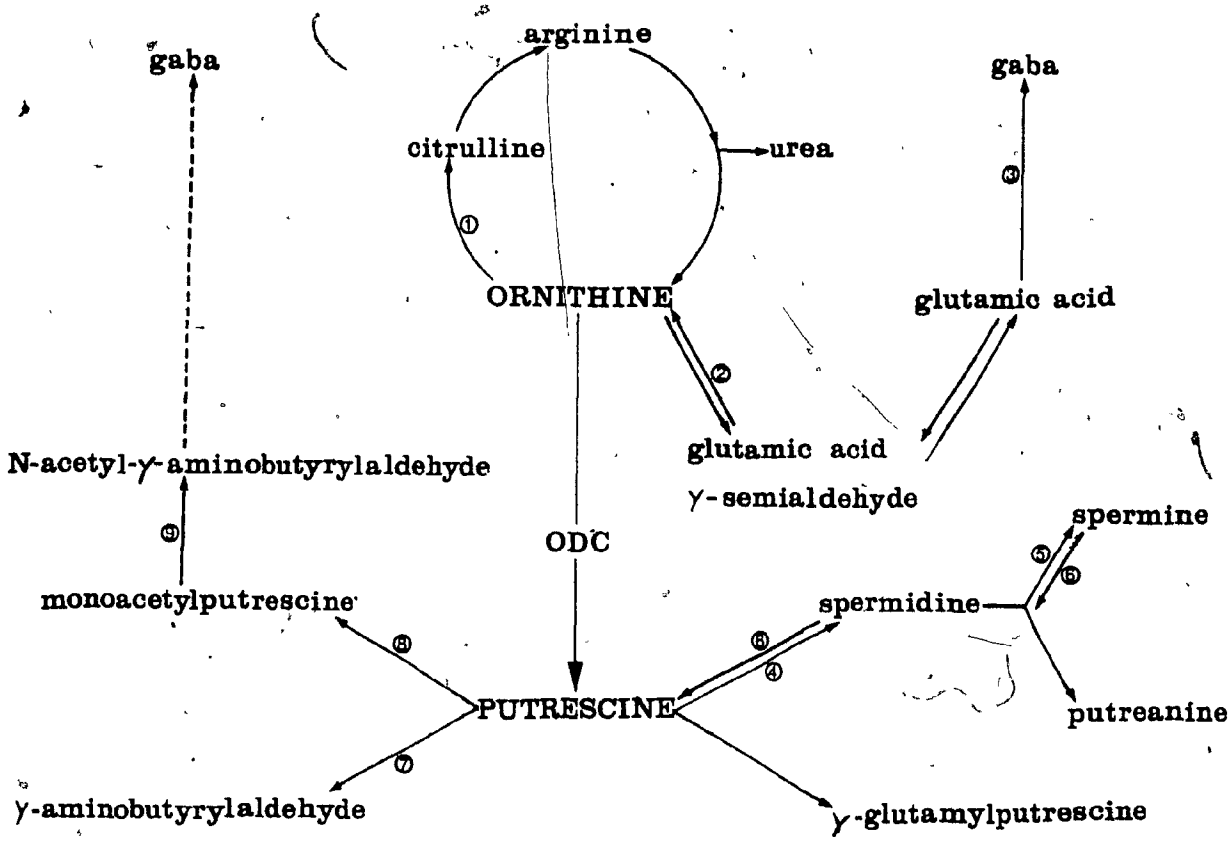


FIGURE 2

Some pathways for L-ornithine and putrescine metabolism in vertebrate tissues. The numbers refer to the following enzymes: 1. Ornithine carbamoyltransferase; 2. Ornithine aminotransferase; 3. Glutamic acid decarboxylase; 4. Spermidine synthase; 5. Spermine synthase; 6. Peroxisomal enzyme; 7. Diamine oxidase; 8. 1,4-Diaminobutane N-acetyltransferase; 9. Monoamine oxidase. The dashed lines indicate that the intermediates have not been fully documented. Adapted from reference 203.

7-day period studied, and this suggests a prolonged half-life (337). In the same system, maximum increase in the polyamine content takes place at 4 h for putrescine; 16 h for spermidine and 64 h for spermine (288).

In mammalian brain, polyamine turnover has been examined after a single intraventricular injection of putrescine (310,305,238,332). Large variations have been reported for the half-life of spermidine in rat brain: 5 days (310,305) and 16-19 days (332), respectively. This has led Antrup and Seiler (7) to do a more extensive study in which endogenous polyamine pools have been radiolabelled by repeated intraperitoneal injection of mice with putrescine, methionine or S-adenosyl-L-methionine for 27 or 57 days. Several interesting conclusions came out of such study: the experimental half-lives of both spermidine and spermine were similar in kidney, skeletal muscle, liver and small intestine (between 11 and 16 days); brain spermidine and spermine exhibited nearly identical biological half-lives of about 42 days; and in brain, the turnover rate of the C_3 moiety of the polyamines from methionine seemed much higher than that of the C_4 moiety from ornithine. This last finding led Antrup and Seiler (7) to suggest that the rate-limiting step in the biosynthesis of polyamines in mouse brain may be the decarboxylation of S-adenosyl-L-methionine and not of L-ornithine.

The actual turnover rate of the polyamines is difficult to determine because of the active interconversion between spermine and spermidine, and between spermidine and putrescine. In rat liver putrescine is formed from spermidine (334), and a peroxisomal enzyme has been recently purified

(139) from the same source which is capable of oxidizing spermine to spermidine and 3-aminopropionaldehyde. The formation of spermidine from injected spermine has also been shown in trout brain (327), goldfish and rat retina (343) and rabbit brain (115). These interconversions can be carried out by a very active oxidase found in ruminant serum resulting in the formation of the cytotoxic aldehyde acrolein (4). Acrolein is a degradation product of the aminopropyl residue.

Information about the degradation of polyamines is scarce. However, an oxidative enzyme has been detected in chick embryo brain (34), apart from the peroxisomal enzyme found in the liver already mentioned. Further evidence for an oxidative process in rat brain is provided by the observation that spermidine is converted to putreanine, the carboxylic acid derived by oxidative deamination of its aminopropyl residue (238). Additionally, spermic acid which is the corresponding derivative of spermine is also found in the brain (146).

Conjugation of putrescine to form monoacetylputrescine has been demonstrated in brain, liver and other organs (7,329). Spermidine and spermine can also be acetylated (22,12,24,331).

Finally, a reaction of considerable interest is the oxidative degradation of putrescine by an amine oxidase pathway (328,330,355) to form the putative neurotransmitter γ-aminobutyric acid (GABA). This pathway (at least in mouse brain) involves both acetylation and oxidation: putrescine → monoacetylputrescine → N-acetyl-γ-aminobutyraldehyde → N-acetyl GABA → GABA (329).

(c) Physiological role of polyamines

Polyamines appear to have little pharmacological activity. Systemic administration of large doses to laboratory animals are very toxic to the kidney (349), and their transport to the brain is restricted by the blood-brain barrier (174). Direct administration of physiological amounts of putrescine into the brain ventricular system has few effects. Spermidine produces sedation and hypothermia. Spermine, which is more potent than spermidine, produces convulsions in mice (6). In large doses the polyamines are very toxic to the CNS; they are distributed by the cerebrospinal fluid and accumulate mainly in the brain stem, where they produce lethal lesions (6). An active transport system appears to be responsible for the redistribution of polyamines in the brain (115). Spermine is also extensively transported in the intact optic nerve of goldfish, but putrescine and spermidine are transported axonally only in regenerating nerve (148).

Because polyamines affect firing of brain stem neurons in cat and rat, it has been suggested that they play some modulator or transmitter function there (333). It is interesting that polyamines are found in both glial and neuronal elements (71). White matter shows much larger concentrations of spermidine than grey matter; and grey matter contains more spermine (174).

A growing field of interest has been the association of polyamines with malignant growth. The presence of elevated polyamine levels in the urine of cancer patients was first reported by Russell and co-workers in 1971 (305), and was later extended to the determination of the polyamine content of other tissue fluids such as serum and cerebrospinal fluid.

The use of polyamine levels as predictive or diagnostic tools in cancer has been the subject of several reviews (304,156,232,92).

Recent reviews have also documented the effects of polyamines on some enzymatic processes, and on the ability of these compounds to interact with and stabilize polynucleotides and various biological membranes and cell fractions (11,156,38). A correlation between spermidine concentration in cells and the amount of ribosomal RNA that is accumulated in them was indicated by early studies (347) and a direct correlation between ODC and RNA polymerase I has been later proposed by Manen and Russell (204,205). As this may be one of the best elucidated functions, the model proposed by these authors will be discussed in the next section.

B. ORNITHINE DECARBOXYLASE: DISTRIBUTION AND CHARACTERISTICS

Ornithine decarboxylase is widely distributed in nature. The discovery of bacterial and plant ODC dates back to the 40s (23); however, the mammalian enzyme was not discovered until 1968 by Pegg and Williams-Ashman in the prostate gland and liver of the rat (266). The same year, two other groups of investigators independently reported that the activity of liver ODC was greatly increased almost immediately after partial hepatectomy (154,306), or administration of growth hormone (158). In general, low ODC activity is found in non-growing situations, whereas high levels are found in cells and tissues undergoing rapid growth and development; such as: in developing chick and rat embryos (306,34,337), developing brain (5,338), rat uterus during the estrous cycle (240),

developing rat retina (195) and developing liver (69); some tumors (306,158, 268,234); and in the cell cycle of large numbers of cell lines (76,81;125, 360). In addition, stimuli such as administration of hormones and drugs, and physiological manipulation result in variable increases in the activity of this adaptive enzyme in liver, heart, brain, kidney, skin, adrenals and other organs (see Table I).

(a) Subcellular localization

ODC is considered a soluble enzyme of the cytosol. In regenerating rat liver, in liver of thioacetamide-treated rats, and in the prostate gland about 90% of the enzyme activity is found in the soluble, cytoplasmic fraction (252,266). The microsomal and mitochondrial fractions have very little or no detectable activity. The specific activities of the nuclear fractions range from one-fifth to one-third of that of the soluble fraction in rat liver, and about one-seventh in the prostate gland.

The subcellular localization of ODC in developing chick embryos is very different from that in the liver and prostate. Thus, 50% of the activity is present in the nuclear fraction, with 5% each in mitochondria and microsomes and the remaining 40% in the soluble supernatant fluid (337). These results are more consistent with the role of the enzyme and of the polyamines in the regulation of nuclear function.

(b) Assay

The standard assay for ODC involves the collection of radiolabelled CO_2 released from carboxyl-labelled ornithine during the reaction. Carbon dioxide is collected by absorption in hyamine hydroxide, protosol or a

TABLE I Response of ornithine decarboxylase to stimuli

Tissue (Stimulus)	Fold increase	Reference
Liver		
Partial hepatectomy (rat)	15-70	
Partial hepatectomy (mouse)	3-4	
Thioacetamide	15-90	
Growth hormone	4-35	
Glucocorticoids	20-60	
Insulin	25	
Glucagon	15	
Thyroxine	10	
Theophylline or dibutryl cyclic AMP	7-10	
Epidermal growth factor	3	
Feeding or infusion of amino acids	8-15	
Hypertonic infusion mannitol	20-30	
Circadian cycles	12	
Celite injection	25-50	
Puromycin	14	
Miscellaneous drugs	2-7	
Combination of triiodothyronine, amino acids, glucagon, heparin	50	
Nafenopin	10	180
Placental lactogen	3	144
12-O-Tetradecanoylphorbol-13-acetate	45	369
Methyl-deficient diet	10-20	226
Kidney		
Growth hormone	5-100	
Glucocorticoids	9	
Unilateral nephrectomy	5	
Epidermal growth factor	8	
Folic acid	6	
Testosterone	200	261
Vasopressin	6	261
Heart		
Growth hormone	2	
Induced hypertrophy	3	
Stress	3	350
α- or β-Adrenergic agonists	4-10	350

(Continued) TABLE I

Tissue (Stimulus)	Fold increase	References
Skeletal muscle		
Growth hormone	2	
Thyroid hormone	4	77
Spleen thymus		
Growth hormone	5-10	
Skin (epidermis		
Epidermal growth factor	4-30	
UV light	6	362
Tumor promoters	6	233
Prostate		
Androgens	6	
Testes		
Gonadotropin	3	
Epidermal growth factor	4-15	
Mammary gland		
Pregnancy	20	
Uterus, oviduct, ovaries		
Estrous cycle	>20	
Estrogens	15-50	
Luteinizing hormone	10	
Gonadotropin	10	
FSH	3	291
Thyroid		
TSH	6	77
Cholera toxin	6	77
Intestine		
Injury	5	194
Brain		
Vasopressin	5	145
Angiotensin II	5	145
NGF	12	145
Electrical stimulation	10	254
Insulin	5	298
6-OHDA	4	15
Several hormones	-	338

Table adapted from reference 231

mixture of ethyleneglycol monomethyl ether and ethanolamine. A micro-assay has recently been described by LaPointe and Cohen (184) which utilized the wells of a spot assay plate to contain the reaction and a $Ba(OH)_2$-soaked glass film filter to collect $^{14}CO_2$. Alternatively, uniformly labelled ornithine may be used as a substrate; the labelled putrescine produced is separated from the substrate either by ion-exchange column techniques (203) or ion-exchange paper (45,60). The last method has the advantage that tritiated ornithine of high specific activity can be used and the assay may be more sensitive than the CO_2 collection method. However, great care must be taken to separate putrescine and ornithine and the method is more time-consuming than the CO_2 method.

A fluorometric assay for ODC has also been described in conjunction with high-performance liquid chromatography (117). Putrescine is partially purified by Cellex P column chromatography and is later converted into the fluorescamine derivative; this is effected in the presence of cupric ion, which inhibits the reaction of interfering amines with fluorescamine. The derivative is separated by reverse-phase chromatography from the polyamines. Again this method is very time-consuming, and large amounts of tissue are necessary for the determination.

(c) Purification: different enzyme forms

Mammalian ODC has been extensively purified from rat ventral prostate (155), from regenerating rat liver (79,166), and from livers of rats previously treated with thioacetamide (252,246). Purification of ODC from crude extracts ranges from 175-fold (79) to 37,000-fold (166), and

many properties of the enzyme are similar in rat prostate and liver.

ODC has a very broad pH-activity curve with an optimum that shifts from pH 7.0 to pH 7.8 as the enzyme is purified (246). Räina and Jänne (154) first noted that increasing the mercaptoethanol concentration from 1 to 10 mM shifted the pH optimum from 7.4 to 8.1.

Rat liver ODC induced by injection of thioacetamide has been separated into at least two fractions by covalent chromatography on an activated thiol-Sepharose 4B. Both forms appear to be dimeric proteins having a molecular weight of approximately 100,000 by equilibrium sedimentation and analysis on a calibrated Sephadex G-200 column. The apparent subunits are approximately 50,000 daltons as determined by electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate (247). Whereas isolated Fraction I is stable, i.e. retained 70% of enzymatic activity after 50 days storage in the presence of 20 mM 2-mercaptoethanol at 4^{o}, Fraction II enzyme lost almost all its activity after storage at 4^{o} for 3 days (246). These two enzyme forms appear to have charge differences as well. The K_m values for ornithine of forms I and II are 0.14 and 0.083 mM, respectively. A purified mixture of the two forms shows K_m values of 0.13 mM for the substrate and 0.25 μM for the cofactor. Both forms exhibit a pH optimum at 7.4 in Hepes buffer and 8.1 in glycylglycine. Antibody against form I is cross-reactive with form II. A single band in the gel electrophoresis analysis of the specific immunoprecipitate corresponding to 50,000 was obtained by Kitani and Fujisawa (167) and Obenrader and Prouty (247). Theoharides and Canellakis (350)

reported that antiserum for ODC precipitates a single polypeptide with a molecular weight of 90,000.

Multiple species of ODC have been separated by chromatography of extracts of rat thymus and kidney on DEAE-Sepharose CL-6B (296). Kinetic evidence for multiple species of ODC has also been obtained for Swiss 3T3 mouse fibroblasts (46), rat prostate (1) and developing heart (185).

(d) Substate and cofactor requirements. Effect of thiols

Prostatic ODC is specific for L-ornithine; it has no activity towards L-lysine, L-arginine or D-ornithine (266). Ono and co-workers (252) tested, in addition to these three amino acids, citrulline, L-glutamine and several derivatives of ornithine on rat liver ODC activity. Of all the compounds studied only D-ornithine showed a small inhibitory action at a concentration of 10 mM. On the other hand, in enzymic preparations from mouse kidney, stimulated with anabolic steroid nandrolone phenpropionate, lysine and ornithine were shown to inhibit the decarboxylation of each other competitively. The K_m values for the decarboxylation were approximately equal to the K_I of the two amino acids, and the maximal pH of decarboxylation was 7.2 for both. Lysine decarboxylation was also shown to occur in rat liver stimulated by growth hormone (270).

Ornithine levels in rat tissues are usually higher than 1 mM (100), whereas the K_m of ODC for L-ornithine is typically 0.1 mM. Therefore, it is unlikely that ornithine availability is a limiting factor in the reaction (213).

The cofactor required by ODC is pyridoxal 5'-phosphate (PLP). It is rather loosely bound to the apoenzyme (266,154,46,270). Unfractionated

tissue homogenates from rat prostate (266) and from the slime mold Physarum polycephalum (229) require the addition of PLP for maximal activity. However, the enzyme in undialyzed liver homogenates (343) and 3T3 mouse fibroblasts (46) contains about 50% of maximal activity without added PLP.

ODC shows no requirement for any metal activator. The metals tested were Mn^{2+}, Ca^{2+}, Co^{2+}, Zn^{2+}, and Fe^{3+} in prostate gland (266) and Mg^{2+}, Ca^{2+} (337) in rat liver. The activity of the enzyme in small intestine is inhibited by the addition of Mg^{2+} (13).

The activity of ODC is preserved by thiols such as dithiothreitol and 2-mercaptoethanol. It is thought that, in the absence of thiols, the enzyme polymerizes to an inactive species. Maximal rates of decarboxylation are obtained when the enzyme extract is prepared, stored, and assayed in the presence of thiols in rat prostate (155), liver (252) and heart (211).

Friedman and co-workers (79) report that ODC activity is strongly dependent on mercaptoethanol concentrations over the range 2-10 mM in partially purified regenerating rat liver preparations, whereas no such dependence is observed with dithiothreitol over the range 1-5 mM. The results of a second group of investigators (252) with partially purified preparations of liver from thioacetamide-treated rats are quite the opposite; thus 5-10 mM concentration of dithiothreitol appears to be more effective in preserving or restoring ODC activity.

(e) Activators and inhibitors

Apart from the well known protective role of sulfhydryl compounds for ODC, the non-ionic detergents Tween 80 and Triton X-100 have recently been

shown to stimulate purified preparations of ODC from rat liver by causing an increase in the velocity of the enzyme (166). Several decarboxylases show an increased rate of decarboxylation in the presence of non-polar solvents (251). The stimulatory effects of serum albumin and the non-anionic phospholipids such as phosphatidylcholine and phosphatidylethanolamine have also been demonstrated by Kitani and Fujisawa (167,168), whereas anionic phospholipids and heparin, as well as synthetic polyanions such as poly-L-glutamic acid and dextran sulfate cause a marked inactivation of the enzyme.

Many compounds inhibit ODC. The major inhibitors acting directly on ODC are substrate analogs such as α-methylornithine and α-hydrazinoornithine (1,156,271). DL-α-Difluoromethylornithine, an enzyme-activated irreversible inhibitor of ODC, is very effective in vivo (10,14,58,73,140, 279). This inhibitor suppresses the increase in uterine ODC activity associated with early embryogenesis in the mouse and arrests embryonic development at that stage (73). Its use as a cytochemical marker is of particular interest (97,98).

The physiologically occurring polyamines, putrescine, spermidine and spermine inhibit the activity of ODC in the rat liver in vivo (152,276), rat intestine (13) and heart (211). Several structurally related but unphysiologic amines, and ethanol have also been shown to inhibit ODC activity (58,108,164,272,275,278). Less specific in vivo inhibition of ODC activity is obtained with compounds that inhibit PLP (252,266); or with inhibitors of protein synthesis such as cycloheximide.

TABLE II Comparison of purified ODC from prostate and liver

Property	Source and type: Rat liver (inducible)	Rat prostate (inducible)
Specific activity	175-37,000-fold	300-fold
K_m, mM	0.2; F_I(0.14), F_{II}(0.083)*	0.1
K_{PLP}, µM	0.25	0.3 K_I(0.06), K_{II}(2)**
Isoelectric point	4.1	
Molecular weight		
Native enzyme	100,000	65-85,000
Protomer	50,000	
Subunits	2	
Inhibitors	Putrescine D-Ornithine, Na, KCl GTP & dGTP L-Lysine Phosphatidylinositol Phosphatidylserine	Putrescine
Activators	Dithiothreitol 2-Mercaptoethanol Triton X-100, Tween 80 Bovine serum albumin Phosphatidylethanolamine Phosphatidylcholine	Dithiothreitol 2-Mercaptoethanol
pH optimum	7.0; 7.4; 7.7	7.2
Reference	79,166,167,168,246,247,252	155,266

* F_I, F_{II} Km value for enzyme fractions I and II

* K_I, K_{II} Km value for enzyme forms I and II

(f) Turnover

The half-life for turnover of ODC activity appears to be the shortest for any mammalian enzyme (337). Russell and Snyder (307) and Hannonen and co-workers (116) showed that when cycloheximide or puromycin, two inhibitors of protein synthesis, are given to either normal rats or to rats 24 h after partial hepatectomy, the enzyme activity decreases very rapidly, with a half-life of 10-11 min. The same half-life was obtained when cycloheximide was administered to weanling rats 3 h after growth hormone administration (310). Inhibitors of RNA synthesis such as actinomycin D given shortly before or at the time of the stimulus prevent the induction of ODC; but one hour after the stimulus they have little or no effect (70,307). From experiments with α-amanitin, Kallio and co-workers (164) have estimated a half-life of 7 h for mRNA of ODC in rat liver. Synthesis of the mRNA appears to take place in 1 h.

(g) Circadian rhythm

It is now well established that ODC activity in rat liver varies over a 6-fold range each day, with peak activity occurring between 8 and 11 pm (122,245). This daily rhythm is altered or abolished in starved animals (122,245), or after they have undergone pinealectomy (316), chemical hepatocarcinogenesis (317), hypophysectomy or adrenalectomy (122). Some of the factors which appear to be involved in maintaining the circadian rhythm of ornithine decarboxylase in liver include: alteration in the lighting schedule (142); availability of food, particularly access to protein (122,142,245); and growth hormone and glucocorticoids (122).

ODC activity in the small intestine of rats having free access to food also shows a marked circadian rhythm, with a peak at midnight (80). The initiation of the circadian rhythm is related to food intake rather than to dark or light (214).

ODC activity of kidney also shows a diurnal variation, which can be modified by pinealectomy (316), and by bilateral adrenalectomy and hypophysectomy (242). No distinct diurnal alterations have been detected in the brain (183), but the pineal gland shows a circadian rhythm with a maximum at midnight (374,375). The rhythm of ODC activity in the pineal gland appears to be determined chiefly by the ratio of enzyme to endogenous inhibitor at different times of the day. This ODC inhibitor is a high molecular weight peptide (30,000 daltons) found in the pineal gland as well as in other organs (76,78,106,127,268).

(h) Ornithine decarboxylase and RNA polymerase I

Manen and Russell (204,205) have proposed a direct relationship between ODC and RNA polymerase, and therefore rRNA synthesis, based on the following observations:

The increased activity of liver ODC, after administration of methylxanthine derivatives is closely followed by an increase in RNA polymerase.

Inhibitors of RNA and protein synthesis which affect the increase in ODC activity, produce a similar attenuation of the increase in RNA polymerase I.

Addition of a partially purified ODC preparation to nuclear or nucleolar preparations increases RNA polymerase I, and it also restores linearity after the polymerase assay has reached the steady state.

Denaturation of ODC preparations by heating at 60° for 10 min results in the loss of ODC activity and abolishes their ability to affect the RNA polymerase I.

Studies utilizing (γ-^{14}C)-ATP and (γ-^{32}P)-ATP indicate that ODC increases RNA polymerase I by affecting the rate of initiation.

ODC has been purified over 2,000-fold by means of an RNA polymerase affinity column.

The conclusion derived from these experiments is that ODC is a rapidly turning-over component of RNA polymerase. The argument presented includes the following facts: (i) a short-lived protein is required for a normal level of transcription of the nuclear genes (75,236), and (ii) in Ehrlich ascites cells, amino acids stimulate the synthesis, or possibly decrease the degradation rate, of this protein (75). Thus, ODC has the same half-life as the labile protein (10 to 20 min) (307), and its half-life can be altered by amino acid supplementation in cells in tissue culture (134).

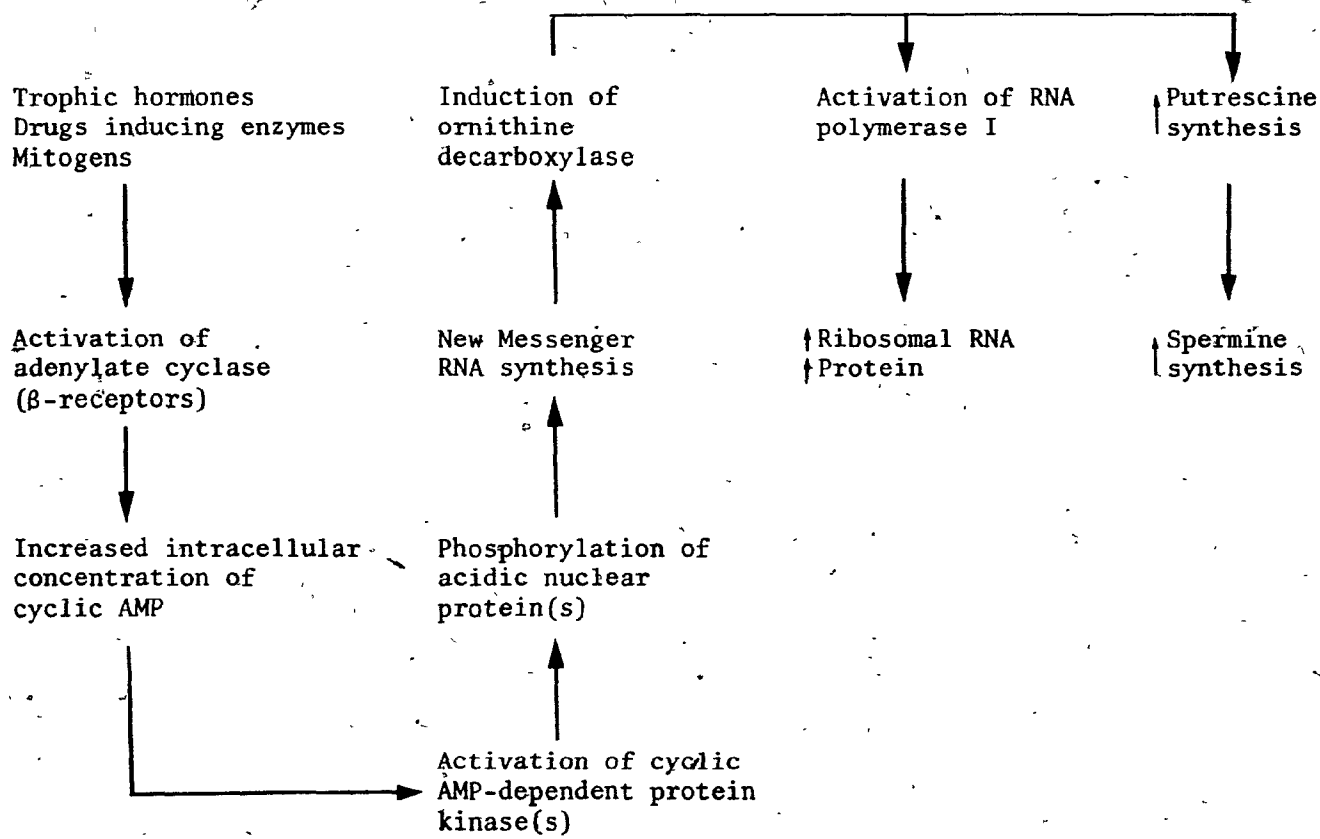
This proposed regulatory function of ODC for RNA polymerase has been integrated into a model of major biochemical events taking place after a trophic stimulus (309). This model proposes that in response to trophic hormones, drug administration, mitogens and various other growth stimuli, there is an increase in the intracellular concentration of cAMP and/or the

TABLE III Temporal increases in cyclic AMP, cyclic AMP-dependent protein kinase activation and ornithine decarboxylase activity in a variety of stimulated tissues

Stimulus	Tissue	cAPM	Protein kinase	ODC
Analogs of cAMP (Dibutyryl cAMP) and/or phosphodiesterase inhibitors	Liver	-	-	-
	Cultured hepatocytes	-	-	-
	Adrenal cortex	-		-
	Adrenal medulla	-		-
	Kidney	-		-
	BHK fibroblasts	-		-
	Glioma cells	-		-
	Neuroblastoma cells	-		-
	H35 cells	-	-	-
	Testis	-		-
FHS, LH	Testis	-		-
TSH	Thyroid	-		-
Regeneration	Liver	-	-	-
Hypertrophy	Heart	-		-
	Adrenal cortex	-	-	-
Glucagon	Liver	-	-	-
	Cultured hepatocytes	-	-	-
Growth hormone	Liver		-	-
	Adrenal cortex		-	-
ACTH	Adrenal cortex	-		-
Isoproterenol	Salivary gland	-		-
	Heart	-	-	-
Cold exposure	Adrenal medulla	-	-	-
Carbamylcholine	Adrenal medulla	-		-
Reserpine	Adrenal medulla	-	-	-
3-Methylcholanthrene or phenobarbital	Liver	-	-	-
Synchrony by mitotic selection	Chinese hamster ovary and V79 cells	-	-	-

Taken from Reference 303.

FIGURE 3 Proposed model of sequential steps in a trophic response



Taken from reference 303

activation of cAPM-dependent protein kinase. This is consistently followed by an elevation in the activity of ODC. There is now some evidence that ODC is induced in parallel with the activation of cAMP-dependent protein kinase (28,29,309). Increased ODC activity in some stimulated tissues is rapidly followed by increased RNA polymerase I activity. This sequence of events has been observed in several systems examined, including some that result in the induction of microsomal mixed-function oxygenases and hypertrophy in rat liver after administration of phenobarbital (33,301), polychlorinated biphenyls (51), and 3-methyl-cholanthrene (33,206,301).

Recently it has been reported that cytoplasmic ODC migrates into the liver nucleus after administration of L-methyl-3-isobutylxanthine to rats, but this increase is not correlated with increased RNA polymerase activity (20). More experimental work of the kind will be necessary in the future before the hypothetical model of Russell and co-workers can be fully evaluated.

C. REGULATION OF ORNITHINE DECARBOXYLASE ACTIVITY

(a) Induction

The same basic pattern of response of ODC activity is observed in many different cell types when they are stimulated to grow or proliferate. After stimulation of enzyme synthesis, there is a lag period of about 1 h, then a sharp increase in activity with a peak occurring around 4 h, after which the activity declines. During the period when ODC activity is declining, the enzyme is refractory to further induction (213).

Increases in ODC activity are brought about primarily by alterations in the rate of protein synthesis. An increased amount of immunoprecipitable protein has been detected in rat liver after growth hormone administration, regenerating rat liver, cultured hepatoma cells and thyroid stimulated in vitro with thyroid stimulating hormone and methyl xanthine (35,138,247, 321). In the majority of the instances the stimulation of the enzyme can be prevented with inhibitors of nucleic acid and/or protein synthesis (231, 285). However, there are cases in which changes in the rate of degradation take place. Prolongation of the half-life of ODC has been reported in a number of cell lines (44,134,280) and in thioacetamide- and carbon tetrachloride-induced ODC activity in rat liver (277). ODC in the liver can also be stabilized following administration of nafenopin, or two competitive inhibitors of the enzyme, α-methylornithine and α-hydrazino-ornithine (180, 217,118).

ODC activity in rat liver shows a biphasic increase after partial hepatectomy, after feeding a casein diet and during early embryonic development (95,198,124). Two different mechanisms have been suggested to operate under these conditions. The first peak is probably under transcriptional control; the second is under translational control and is dependent on the RNA formed during the initial reaction. Studies with enucleated cells produced by cytochalasin B have shown that cytoplasts or cytoplasm freed of nuclei still respond to growth stimuli with an increase in ODC (285). ODC activity in the enucleated cell must be controlled at a translational level.

(b) Multiple enzyme forms

Multiple active and inactive forms of ODC have been isolated from various tissues. This has raised the possibility of the participation of such forms in the regulation of the enzyme. Thus, Swiss 3T3 mouse fibroblasts have two different forms of the enzyme with different K_m for the cofactor. Upon stimulation of the cells by growth hormone conversion of the less active to the more active form takes place while ODC activity is increased 10-fold (46). Similarly, a recent report indicates that ODC in hormonally stimulated adult heart and in non-stimulated neonatal heart has a lower K_m for the substrate than that of non-stimulated adult heart (185). These changes are not accounted for by alterations in soluble factors which could influence the enzymic activity. Finally, active interconversion of the two enzyme forms in Physarum polycephalum takes place rapidly after stimulation with cycloheximide. This drug does not cause a loss in ODC activity, but rather induces a sharp change in the ability of this enzyme to bind PLP (229). A protein factor is involved in the interconversion of the alternate states of ODC (227,228). This factor appears to be a heat-labile, acidic protein with a molecular weight of about 35,000 which binds to macromolecules in crude fractions isolated using low ionic strength buffers.

(c) Product regulation

Administration of diamines in vivo at the time of partial hepatectomy blocks the induction of ODC that usually ensues. This suggests that the enzyme is controlled by a repression type of mechanism(152,276). The mechanism of regulation of ODC by the product of the reaction is quite

complex. It is associated with a decrease in the amount of immunoprecipitable enzyme protein (164). Additionally, putrescine and other polyamines promote the synthesis of a protein inhibitor, or antienzyme, that specifically interacts with ODC to form an inactive complex. This inhibitor has been detected in several cell lines (47,76,128,217), in rat thyroid and liver (127,78,160,268,277,36), and in chicken liver (106). It has a molecular weight of 26,000 daltons, is heat-labile, and loses its activity by treatment with chymotrypsin or trypsin.

These two polyamine regulatory mechanisms for ODC appear to be concentration-dependent. That is, small concentrations favor suppression of ODC activity while large concentrations promote the synthesis of the antienzyme (19,219). Heller et al. (129) and Canellakis et al. (37) have suggested that the cell membrane contains a critical regulatory site for ODC, and that at low levels of extracellular polyamines, the induction of ODC activity can be inhibited through these membrane-mediated sites. In line with this hypothesis, agents that affect the cell cytoskeleton such as colchicine, cytochalasin and vinblastine inhibit the induction of ODC (42).

Two post-translational modifications of ODC have recently been reported which result in inhibition of the enzyme in vitro: putrescine binding to ODC by a transglutaminase (302), and phosphorylation of ODC by a polyamine-dependent protein kinase (8).

In summary, proposed mechanisms for the regulation of ODC include transcriptional, translational and post-translational models. Furthermore,

some evidence suggests that different inducers may affect different aspects of the control of ODC activity in the same system (49,186).

D. ORNITHINE DECARBOXYLASE IN THE ADRENAL GLAND

(a) Hormonal effects

ODC activity in the adrenals of hypophysectomized rats is markedly increased by the administration of ACTH (189,190,297). These effects are not secondary to stimulation of steroidogenesis: although hydrocortisone stimulates activity of the enzyme in the liver and kidney it has no effect on adrenal ODC (297); and inhibition of adrenal steroidogenesis by aminoglutethimide does not interfere with the increase in ODC activity following ACTH stimulation (190). These two events are also uncoupled in a mutant of ACTH-responsive mouse adrenocortical tumor cell line Y1 (176).

Stimulation of ODC by ACTH is preceded by increases in adrenal cAMP content; administration of dibutyryl cAMP to rats increases ODC activity as well (297). Although Richman et al. (297) have reported a close correlation between the dose of ACTH, the increase in cAMP concentration, and the activity of ODC in the adrenal, Levine et al. (190) were not able to reproduce this dose-dependent relationship. In vitro studies with adrenocortical tumor cell clones indicate that the action of ACTH on ODC activity requires the participation of adenylate cyclase, cyclic AMP, and cAMP-dependent protein kinase (176). A close relationship between an increased activity ratio of cAMP-dependent protein kinase and the induction of ODC has also been shown in the adrenal gland stimulated to grow by

unilateral adrenalectomy (32).

The increases in ODC activity produced by ACTH administration are prevented by administration of cycloheximide; in this way, sustained new protein synthesis appears to be required for ACTH to stimulate adrenal ODC activity. Actinomycin D, on the other hand, blocks the effect of ACTH 16 h after hypophysectomy but not 1 h after the operation (190). Therefore, it has been suggested that ACTH stimulates ODC activity by a post-transcriptional mechanism in the acutely hypophysectomized animal, whereas in the animal deprived for several hours of pituitary hormones, ACTH also stimulates transcription of new mRNA which is involved in the regulation of ODC (190,297).

Growth hormone administration to hypophysectomized rats results in an increase in adrenal ODC activity (189,242). This effect is not preceded by increases in adrenal cAMP content (189). Thus, growth hormone and ACTH appear to stimulate ODC activity by different mechanisms, one cAMP independent, and the second dependent. This is further supported by their synergistic effect when administered together (189).

Another trophic hormone, prolactin, causes large increases in ODC activity of intact and hypophysectomized female rats, and in intact male rats. These effects are dose-related and age-dependent (295,354).

Administration of nerve growth factor (NGF) into the cerebroventricular system of the rat induces ODC activity in the adrenal gland. Hypophysectomy or pituitary stalk section inhibits the increase indicating that NGF causes an activation of the hypothalamo-hypophyseal endocrine system (237). The direct action of NGF on a clonal cell line (PC_{12}) from a transplantable

rat adrenal pheochromocytoma has also been demonstrated to induce ODC activity (121,143). These increases in ODC activity appear to be independent of the ability of NGF to promote neurite outgrowth (102). Most of the reports support the concept that cAMP mediates the NGF induction of ODC in PC12 cells (178,326). Induction of ODC activity by NGF in superior cervical ganglia of rats *in vivo* and *in vitro* appears to be mediated by a cAMP-dependent mechanism as well (196), and occur through two types of receptors, one on the cell membrane involving cytoskeletal structures (179), and the other intracellular (130).

(b) Neuronal effects

Several stimuli that cause alterations in neuronal function can increase ODC activity in the rat adrenal medulla. Among these are application of the stressors cold exposure (28,290) and immobilization (290), and administration of drugs such as methylxanthine derivatives (29), reserpine (31,59,290), and cholinergic agonists (289,303). These effects are prevented by denervation of the adrenal, and mecamylamine (a ganglionic blocker) lowers the increase in ODC produced by carbamylcholine (303). The muscarinic agonist, oxotremorine, stimulates the enzymic activity mainly by a central mechanism (289). This has been demonstrated both by the failure of methylatropine, a peripheral muscarinic blocker, to prevent the effect of oxotremorine, and by the reduction of the oxotremorine increase in animals with the spinal cord transected.

The increases in ODC activity in the adrenal medulla are always preceded by elevated cAMP levels, but there is no direct correlation between the absolute increases in cAMP and ODC. The participation of a

cAMP-dependent protein kinase in the transynaptic induction of ODC is, however, suggested by the close correlation between the degree of activation of the two enzymes after reserpine administration and cold exposure (31). Experiments with actinomycin D and cycloheximide seem to indicate that de novo protein synthesis of ODC from newly transcribed mRNA is responsible for the increased activity of this enzyme (31).

The stressors cold exposure (31,290) and restraint (290), as well as the administration of reserpine, aminophylline and oxotremorine (59,20, 29,290) increase adrenocortical ODC activity. Similarly to the adrenal medulla, an increase in cAMP content is observed in the cortex, and the effect of cold exposure and aminophylline is abolished by cycloheximide and actinomycin D (31).

The action of oxotremorine on adrenocortical ODC activity is prevented in hypophysectomized rats; this demonstrates that its action is ultimately mediated by the pituitary gland (289), probably by the release of ACTH. The cortical response to the stressors and reserpine is expected to be mediated also by the pituitary since the three are known to activate the hypothalamo-pituitary axis.

E. THE ADRENAL GLANDS

The adrenal glands are located close to the upper pole of each kidney. They are formed by two distinct endocrine tissues, the medulla and the cortex, covered by a thin connective tissue capsule. The cortex which constitutes about 75-90% of the gland in most mammalian species surrounds the medulla completely. These two parts are different in origin, structure

and functions.

The chromaffin tissue consists of cells derived from ectoderm, and the cortical tissue is composed of cells derived from the mesoderm. The two tissues associate during development to form the adrenal glands.

(a) Blood supply

In the rat, the main blood supply is derived from an artery that leaves the central aspect of the abdominal aorta rostral to the respective adrenal gland, and usually supplies the upper pole of the gland. The second major source is a vessel that arises from the aorta at a more caudal level and supplies the medial aspect of the gland (53). The cortex is particularly rich in blood supply. The arteries to the adrenal break up into many branches as they approach the gland and penetrate the capsule at different points, forming the capsular plexus of arterioles in the zona glomerulosa. From here, capillaries cross the cortex and continue into medullary sinusoids which converge at the central suprarenal vein. Thus, some of the capillaries from the capsular plexus end in the internal layers of the cortex without reaching the medullary vessels, while larger vessels, the "arteriae medullae", may penetrate the cortex without branching and terminate directly in the medulla (120).

(b) Innervation

The adrenal medulla is supplied predominantly by preganglionic neurons. In the rat, small nerves leave the greater splanchnic neurons, and accompany the superior adrenal artery to the gland. Usually the lesser splanchnic, and infrequently, the lumbar sympathetics, supply a few fibers (197). These preganglionic sympathetic fibers pass through

the adrenal cortex in bundles and enter the medulla to terminate directly, once they have ramified, upon individual chromaffin cells (137,197). It has been suggested that the NE- and E-containing cells have separate innervation (68). Recent evidence for the two types of fiber innervating the adrenal in the rabbit has been provided by the electrophysiological work of Hirano and Niijima (131).

Studies concerning adrenocortical innervation are not so consistent. Thus, some early studies based on light microscopic techniques failed to detect cortical innervation (137,197), while others have reported direct nervous termination upon rat cortical cells (187,225). Myelinated nerves have been noted in the subcapsular region by electron microscopy (244), and in close proximity to the capsular fibroblasts of the mouse (224) and rat (256). In summary, several studies have demonstrated that autonomic nerve terminals lie in close proximity to parenchymal cells of various cortical regions and form a network around small arteries. All these fibers are much finer than the myelinated fibers to the medulla.

(c) Histology and function

Histochemical and electron-microscopic investigations have determined that there are two types of parenchymal cell in the adrenal medulla. One type selectively stores and secretes NE, the other, E (68,188,241,52). The majority of cells in the adrenal medulla of adult rats are E-containing and are arranged as small islands distributed through that tissue (361). In addition, cortical islands are found in the medulla in close relationship to NE cells (241).

The adrenal cortex is composed of three concentric zones distinguished by the grouping of the cells: (i) the zona glomerulosa, lying against the outer connective tissue capsule; (ii) the zona fasciculata or middle layer; and (iii) the zona reticularis, adjacent to the medulla.

In rats, a sudanophobic layer (intermediary zone) can be delineated between zona glomerulosa and zona fasciculata (123).

The zona glomerulosa secretes mainly aldosterone, a mineralocorticoid having the prime function of maintaining electrolyte balance. The factors that regulate aldosterone secretion include the renin-angiotensin system, and sodium and potassium balance (253); secretion of aldosterone is relatively independent of pituitary ACTH control. The two inner zones secrete glucocorticoids and adrenal androgens. In man, cortisol is the major glucocorticoid product, but in laboratory animals such as the rat or the rabbit, it is corticosterone. Corticosteroids play a prominent role in the metabolism of carbohydrates; and the secretion of these hormones depends upon intact function of the hypothalamo-pituitary adrenocortical axis.

F. HYPOTHALAMO-PITUITARY ADRENOCORTICAL SYSTEM

(a) Regulatory mechanism of the adrenal cortex

Adrenocortical secretion of glucocorticoids is controlled primarily by the anterior pituitary hormone adrenocorticotropin (ACTH). This polypeptide was partially purified and standardized in the late 1940s (335); and its 39 amino acid sequence first analyzed by Bell in 1954 (16).

ACTH was synthesized in the 1960s (132,193,324) and has been later purified in many species. Evidence derived mainly from studies involving bio- and radioimmunoassay points to three different forms of ACTH. They differ in molecular weight and in biological activity (372). Coslovsky and Yalow (50) relate the ratio of cortisol/corticosterone production to the form of ACTH present in mammals. That is, species whose predominant glucocorticoid is cortisol contain the smallest form of ACTH, whereas the intermediate form of ACTH is found in mammals (rat, mouse) whose predominant adrenocortical steroid is corticosterone. The three forms of ACTH have been detected in the pituitary of rats and mice (199,181); their presence is not restricted to the anterior lobe, for they also occur in the intermediate-posterior lobe. It has been proposed that the distribution of the multiple molecular-weight forms of ACTH over the various parts of the pituitary might be related to physiological function. Thus, the intermediate lobe of the pituitary, being innervated by the hypothalamo-hypophyseal tract, may be preferentially depleted of ACTH by neurogenic stimuli (199), whereas the release of ACTH in the anterior lobe is regulated by hypothalamic releasing factors. However, we do not know yet the physiological significance of the ACTH in the intermediate lobe. In fact, it has been demonstrated (104) that the intermediate-posterior lobe does not secrete functionally significant quantities of ACTH into the circulation.

(b) ACTH regulation: the hypothalamo-pituitary unit

Secretion of ACTH from the anterior pituitary is controlled by the central nervous system (CNS). Regulation centres first of all in

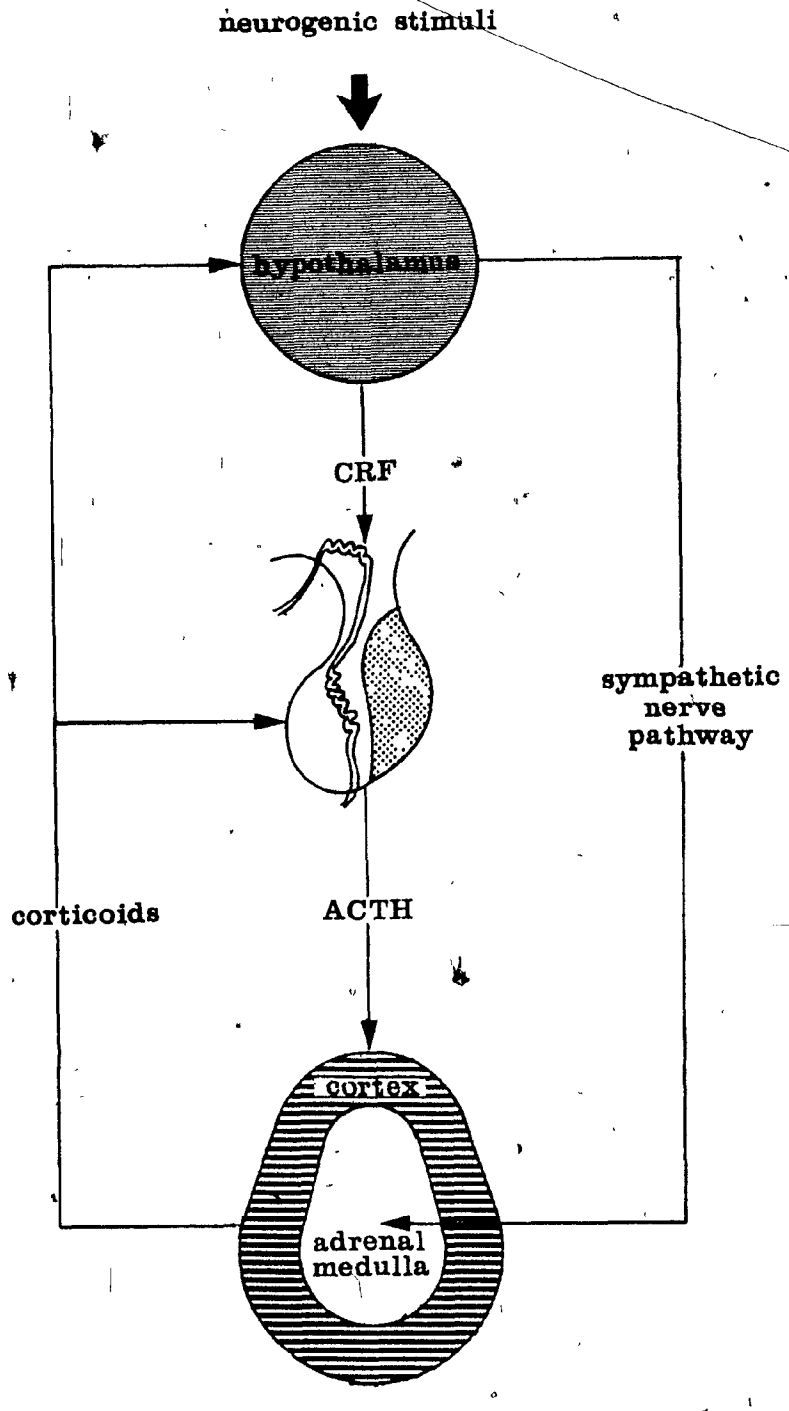


FIGURE 4.

Schematic diagram of the hypothalamo-pituitary-adrenal system

the hypothalamus. The classical neurohumoral mechanism proposed by Harris (119) involves hypothalamic-hypophysiotropic hormones which are released by the nerve terminals in the median eminence and proximal part of the pituitary stalk. These substances enter the capillaries of the portal vascular system and are carried by the vessels to the pituitary. The presence of an ACTH releasing factor (corticotropin releasing factor, CRF) was reported in hypothalamic extracts by Saffran and Schally (312), and Guillemin and Rosenberg in 1955 (110), and in posterior lobe extracts (312). The latter factor was demonstrated to be vasopressin (216). Because animals with hereditary diabetes insipidus, lacking vasopressin, could release ACTH, it was clear that vasopressin was not the only CRF, and that CRF from hypothalamic extracts was probably the major one (218, 215). Several studies have also demonstrated the presence of CRF activity in tissue of extrahypothalamic origin (25). The chemical structure of an ovine hypothalamic peptide that stimulates secretion of corticotropin and β-endorphin both *in vivo* and *in vitro* has been recently described (357). The immunocytochemical localization of this peptide will undoubtedly be elucidated soon. CRF-containing cells appear to be localized in the mediobasal hypothalamus (MBH) (181). This is termed the hypophysiotrophic area by Halász et al. (113). When this part of the hypothalamus is surgically separated from the rest of the brain, its CRF content remains constant or actually increases (175).

(c) Neural pathways to hypothalamic-pituitary ACTH release

Agents playing a role in ACTH release can reach the pituitary gland in two ways: humorally or neuronally. The humoral pathways are all those

that lead to the ACTH-producing cells without involvement of the CNS, i.e. via the blood vessels of the pituitary gland. The neural pathways include all the stimuli-activated neurons, i.e. paths that require the integrity of the CNS for exerting their effect (255).

Much effort has been expended in attempting to elucidate the neural pathways mediating the release of ACTH in response to stressors. The most commonly used techniques include destructive lesions and focal stimulation of areas of the CNS. For example, it has been shown that the release of ACTH following traumatic stress to a limb depends on the integrity of a neural pathway ascending towards the hypothalamus. Section of the peripheral nerves (62) or of the spinal cord (207) blocks ACTH release normally following fracture of the leg. Transection of the cord also prevents the releasing effect produced by other stressful stimuli (62,292,202). Hemisection of the cervical spinal cord, and lesions of the lateral column induce a long lasting inhibition of the ACTH releasing effect produced by the contralateral leg fracture, showing that the pathways conveying ACTH releasing information cross the midline (202,200). Removal of most of the forebrain, leaving a large hypothalamic peninsula, does not block the response to a leg break (207,208) or to immobilization (61), although further removal of the thalamus and midbrain greatly diminishes the response to either stress. Disruption of the hypothalamo-pituitary link by extensive electrolytic lesions blocks the response to surgical trauma (25). Finally, the results of complete deafferentation of the MBH, as well as of various types of incomplete hypothalamic deafferentation (202,201,103,257), have led Palkovits et al. (257) to postulate

that the adrenocortical response to surgical trauma depends on the integrity of a well-circumscribed region of the hypothalamus: the lateral-basal region of the retrochiasmatic area.

Nervous structures outside the MBH also exert control over the anterior lobe of the pituitary; Halász and Pupp (112) first termed them "the second level of control". Halász et al. (114) have later observed that after complete deafferentation of the MBH basal corticosteroid levels are not only maintained but even elevated. They suggest that the lesions eliminate inhibitory influences over ACTH secretion. Other inhibitory factors from various areas of the CNS (63,65,66,274,339,340,344), as well as stimulatory ones (66) have been proposed on the basis of stimulation and lesion experiments. More recent electrophysiological investigations in the rat hypothalamus have provided additional support for this postulate (294). Thus, electrical stimulation of different parts of the amygdala leads to enhanced or diminished excitability of tuberoinfundibular neurons located within the ventromedial nucleus.

In summary, it is generally accepted: (i) that there are multiple anatomically segregated parallel pathways that converge on the median eminence; and (ii) that the release of ACTH may be adjusted by the integration of the facilitatory and inhibitory signals from these pathways (111,255).

(d) Monoaminergic innervation of the hypothalamus

(i) Functional anatomy

The distribution of monoamine neurons in the hypothalamus has been extensively studied by histochemical, ultrastructural, and enzymatic-

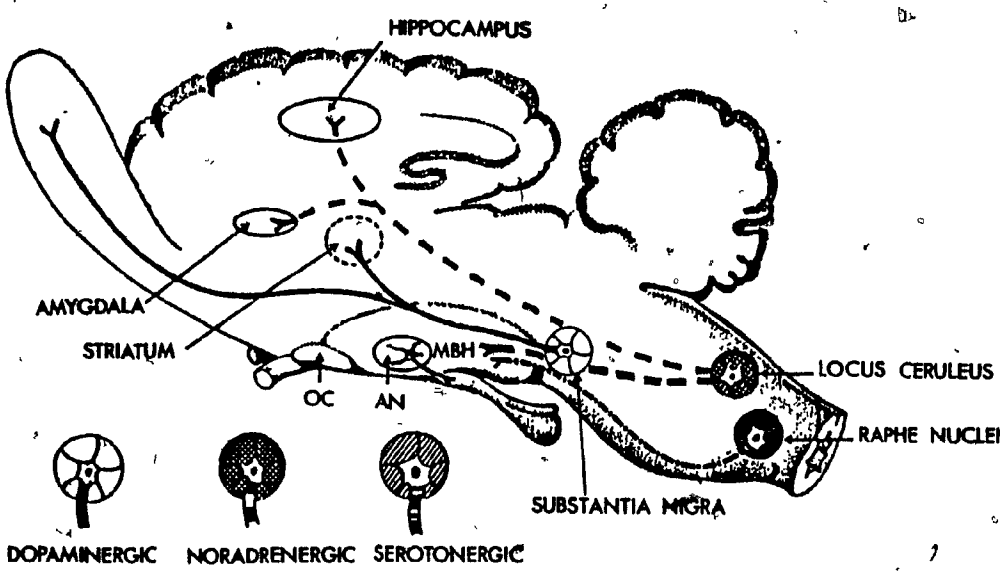


FIGURE 5

Monoaminergic pathways in mammalian brain. The principal location of the neurons containing norepinephrine, dopamine, and serotonin is in the mesencephalon and pons. Abbreviations: MBH, mediobasal hypothalamus; AN, arcuate nucleus; OC, optic chiasma. Adapted from reference 111.

isotopic techniques. Dopamine occurs in the hypothalamic nuclei (258, 367) in different concentrations, and functions in two systems organized at different levels: those located entirely within the hypothalamus; and those responsible for interstructure communication. The main, intrinsic dopaminergic system is the tuberoinfundibular tract described originally by Fuxe (84). Its cell bodies are located in the arcuate and paraventricular nucleus and the fibers terminate in close proximity to hypophyseal portal vessels in the external layer of the median eminence (85,135). A second intrahypothalamic pathway, designated as the incertohypothalamic system, originates in the parafascicular thalamic nucleus and medial zona incerta (group A_{11} and A_{13}) and projects to dorsal and anterior hypothalamic areas (88,21,149). A rostral part originates in A_{14} and is formed by a few cells in the periventricular preoptic nucleus (21). Finally, dopaminergic terminals of extrahypothalamic origin are also found in the median eminence. Their perikarya originate in the A_8, A_9 and A_{10} cell bodies of the substantia nigra and ventral tegmental area (55). Lesions of this region result in degeneration of axons (256) and loss of about half of the dopamine (DA) content in the median eminence (169).

All cell groups of the hypothalamus receive noradrenergic axons (258,267). Unlike DA-containing neurons, there appear to be no NE-containing perikarya within the hypothalamus. Thus, all NE within the hypophysiotrophic area is contained within nerve terminals, the cell bodies of which lie in the brainstem and areas A_1 and A_7 of Dahlström and Fuxe (55). After lesions in the locus coeruleus, terminal degeneration of fibers (377) occurs in parallel to the decrease of NE content (170,299)

in several hypothalamic nuclei.

All hypothalamic cell groups contain 5-hydroxytryptamine (5HT) with particularly high concentrations in the arcuate and suprachiasmatic nuclei and the median eminence (84). Like NE, 5HT in the hypothalamus is localized within the nerve axons and terminals whose cell bodies reside in the brainstem (84,256). A large part of the fibers arise from the dorsal raphe nucleus of the midbrain, as deduced from the biochemical and morphological changes brought about by electrolytic lesions of this cell group (256,3). However, Geyer et al. (96) and Jacobs et al. (150) report approximately equal innervation of the hypothalamus by the dorsal and medial raphe nuclei. Moreover, Vernikos-Danellis et al. (364) suggest that the hypothalamus receives only a small portion of its fibers from the dorsal raphe. They measured ^{3}H-5HT uptake and tryptophan hydroxylase activity in the hypothalamus of rats with lesions of the dorsal or medial raphe nucleus. Recently, a group of intrahypothalamic serotonergic neurons, with cell bodies in the dorsal medial nucleus, has been identified (87,74).

In addition to DA, NE and 5HT, E-containing axon terminals have been observed in the hypothalamus (136,358). Cholinergic neurons are also found (26,41,149,260)

(ii) Role of monoamines in regulation of ACTH secretion

The relation between neurotransmitter content of discrete aminergic tracts and changes in neuroendocrine activity was first demonstrated by Fuxè and Hökfelt (86), and involved the tuberoinfundibular system. The most clear-cut involvement of an amine in adenohypophyseal regulation

is represented by the interaction of this dopaminergic system with prolactin release (172). This hormone is under inhibitory control of the hypothalamus (243,222), and DA acts as a prolactin inhibitory factor (221, 105,320,67,107). In contrast, NE and 5HT seem to have an excitatory role in prolactin release (173).

In regard to ACTH secretion, it is not clear what sort of neurotransmitter is involved (111). Pharmacological studies in cats suggest that NE may tonically suppress the release of ACTH (315,90). In rats, the available evidence for interaction of NE with ACTH regulation is contradictory. Intraventricular administration of NE to unanesthetized rats decreases plasma corticosteroids, but the amine fails to block the stress-induced release of ACTH (111). According to Ganong (90) and Fuxe et al. (89) DA does not play a very important role. However, the DA content of certain MBH cell groups decreases upon application of stress stimuli, whereas it does not change in other brain regions studied (259). Immobilization stress for more than 3 h also results in an increased DA turnover in the median eminence (325). Administration of apomorphine and other DA agonists to male rats also increases the levels of circulating steroids (171,83). Thus, a role for DA cannot yet be excluded.

Cholinergic synapses appear to be involved in the stimulation of the adrenocortical system (255). Implantation of atropine into various hypothalamic areas inhibits CRF and, consequently, ACTH release (126), whereas intrahypothalamic or intraventricular injections of carbachol stimulate ACTH release (2).

The literature concerning the role of brain 5HT in tonic regulation of hypothalamic-hypophyseal-adrenocortical systems, as well as changes occurring under stress, also contains inconsistencies. Some pharmacological studies tend to support an excitatory role (223). Thus, direct injection of 5HT into the ventricular system of the rat brain or into different brain areas produce adrenocortical activation (2,239). The systemic administration of the serotonergic drugs, 5-hydroxytryptophan, fluoxetine and quipazine does the same (82,273,364). The in vitro studies of Jones et al. (163) and Buckingham and Hodges (27) support this excitatory role. Jones et al. (163) have found that the release of CRF from incubated rat hypothalamus is stimulated by 5HT in a dose-related manner and that this stimulatory action is antagonized by methysergide. Furthermore, reduction of cerebral 5HT function by electrolytic lesions of the medial raphe nucleus or intraventricular administration of 5,7-dihydroxytryptamine blocks the stimulatory role of d-fenfluramine or corticosterone release (322). On the other hand, intracerebral 5HT blocks stress-induced ACTH secretion (363), whereas p-chlorophenylalanine administration facilitates the stress response (364). Because of such results one may postulate an inhibitory role for 5HT in ACTH release. In parallel, inhibition of synthesis of the amine elevates resting plasma levels of corticosterone (318,300). These latter effects are related to the well known role of serotonergic neurotransmission in the control of circadian periodicity of adrenocortical as well as other anterior pituitary hormone secretions (319,172).

(e) Regulation of ACTH secretion by adrenal glucocorticoids (Feedback)

Among the humoral factors regulating the secretion of ACTH the best known are the adrenal glucocorticoids. Their influence was first demonstrated by Ingle et al. (147), who produced adrenocortical atrophy by administration of adrenocortical extracts to intact rats. Injection of anterior pituitary extracts did not do so. In later studies, Sayers and Sayers (314) have shown that the adrenal response to a given stimulus is reduced by the prior adminsitration of corticosteroids, and that this effect is proportional to the amount of steroid given. It is well established now that there are two temporally distinct periods of corticosteroid feedback inhibition on ACTH release in response to stress. The first, fast feedback inhibition, is of short duration and occurs within the first 20 min following administration of corticosterone (314,56); inhibition of ACTH secretion is related to the rate of change of plasma corticosterone levels rather than to the absolute plasma concentration of the steroid and is saturable (161). The second, delayed or slow feedback, occurs several hours after the stimulus when the plasma corticosterone is declining or low, and it is proportional to the concentration of corticosteroids in the blood (336).

The sites for the feedback effect of glucocorticoids are still unclear. There is evidence for a direct action on the pituitary, on the hypothalamus, and for an effect on mesencephalic and limbic-system structures. Thus, corticosterone, cortisol and dexamethasone suppress the ACTH release by the rat anterior pituitary tissue both at the short and delayed feedback

levels (313). The three steroids act also at the level of the hypothalamus to inhibit CRF synthesis and secretion (163). In experiments with basal hypothalamic-lesioned rats, Jones et al. (162) demonstrate that the biphasic inhibitory response to corticosteroids occurs both at the pituitary and hypothalamic level. Other in vivo experiments include administration of steroids to intact rats after application of a steroid-sensitive stimulus (57) and implantation of steroids in different brain regions (220).

In addition to the feedback action of the target gland hormone, termed external or "long-loop" feedback, there is experimental evidence that ACTH itself may influence the synthesis and release of CRF by the median eminence (326). This effect has been termed the "short-loop" feedback. The recent demonstration by Bergland et al. (18) that pituitary ACTH is directly carried to the brain from the pituitary in the sheep gives support to the second feedback mechanism. Furthermore, an ultra-short feedback in which CRF regulates its own production has been postulated based on the assumption that the brain contains receptors sensitive to circulating levels of releasing factor.

In summary, afferent neural inputs provided by different environmental stimuli (stressors) activate the pituitary adrenal system. A component of the hypothalamic response to this neural input is the release of CRF by the neurosecretory cells of the hypothalamus. CRF is carried to the anterior pituitary through the portal veins where it causes release of ACTH into the systemic circulation. Blood-borne ACTH from the pituitary stimulates cells of the adrenal cortex to synthesize and release

glucocorticoids. Adrenal steroids, in turn, exert a feedback effect and therefore regulate release of ACTH from the pituitary.

G. CENTRAL NERVOUS PATHWAYS REGULATING THE SYNTHESIS OF CATECHOLAMINES IN THE ADRENAL MEDULLA, AND THEIR RELEASE

The immediate stimulus for discharge of catecholamines from the adrenal medulla is the liberation of acetylcholine at the preganglionic nerve terminals where it activates mainly nicotinic receptors. The presence of muscarinic receptors (165) and their participation in the release of E after electrical stimulation of the splanchnic nerve has been demonstrated in the adrenal medulla of the rat (376). Acetylcholine then causes depolarization of the plasma membrane mainly by the inward movement of sodium and calcium. The influx of calcium is believed to be the main stimulus responsible for the mobilization of the catecholamines for their secretion.

Increased E secretion of the adrenal medulla is one of the body's first responses to emotional and physical stress as has been classically demonstrated by Cannon (39). Thus, cold exposure, immobilization, ether, insulin-induced hypoglycemia and glucagon are but a few of the stimuli causing release of E (192). CNS mechanisms for the regulation of adrenal medullary secretion have been identified by direct electrical stimulation of the medulla oblongata, hypothalamus and other brain structures in cats and dogs (368,293,101), and in the rat (209,210). Moreover, the increase in E secretion caused, for instance, by the administration of insulin is dependent on an intact nervous connection of the adrenal medulla with the

CNS (17,54,40).

Environmental stimuli, such as cold exposure and immobilization (177,281), and the administration of certain drugs, such as reserpine (235,352), aminophylline and carbamylcholine (109), and oxotremorine (191), cause a delayed long lasting induction of adrenal tyrosine hydroxylase. This induction of the rate-limiting enzyme for catecholamine biosynthesis is mediated transynaptically (353). Similarly, dopamine-β-hydroxylase, the enzyme catalyzing the conversion of DA to NE can be increased by the same stimuli (230,9). Furthermore, the neural regulation of adrenal tyrosine hydroxylase involves a central excitatory dopaminergic and an inhibitory serotonergic system, arranged in a sequential manner (282). The DA system originates rostral to the thoracic spinal cord (93). The serotonergic system originates partly in the medial raphe nucleus (282). The existence of a second descending serotonergic pathway is supported by drug experiments in spinal rats (94,341).

Investigations demonstrating the transynaptic induction of adrenal tyrosine hydroxylase by agents that cause an increase of impulse flow in the afferent innervation, in particular, the work of Quik (281,282,283) et al. have served us as a model for the present studies of pathways involved in the regulation of adrenal ODC activity. Because dopaminergic agents activate both the sympathetic nervous system (281,282) and the hypothalamo-pituitary-adrenocortical systems (83,99,171), we are exploring in this thesis the mechanism of regulation of the adrenomedullary and adrenocortical enzymic activities.

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CHAPTER II

Effect of Apomorphine, Piribedil and Haloperidol on Adrenal Ornithine Decarboxylase Activity of the Rat

SUMMARY

The administration of the dopaminergic drugs apomorphine and piribedil to rats results in an increase in the activity of ornithine decarboxylase of the adrenal medulla and cortex. Pretreatment of the rats with the dopamine-receptor antagonist haloperidol causes a partial blockade of the apomorphine-induced effect at 4 h in both adrenal medulla and cortex. At 6 h, however, haloperidol does not block the effect of apomorphine and produces an increase in ODC activity of both structures when administered alone. Hypophysectomy abolishes the cortical ODC response to apomorphine and haloperidol, and the medullary response to haloperidol. The results suggest that the response of cortical ODC activity to apomorphine and haloperidol is entirely mediated by the hypophysis, and that the effect of apomorphine and the antagonistic action of haloperidol towards apomorphine in regard to the induction of adrenal medullary ODC must be taking place at some central site independent of the hypothalamic-hypophyseal system.

The kinetic studies suggest that the adrenal gland contains two different forms of ODC with different constant of affinity for the cofactor and similar constants of affinity for the substrate. The increase in ODC activity produced by apomorphine is dependent upon protein synthesis de novo.

INTRODUCTION

Ornithine decarboxylase (EC 4.1.1.17, ODC), the rate-limiting enzyme in polyamine biosynthesis (59), catalyzes the conversion of ornithine to putrescine. Mammalian ODC is characterized by an extremely short biological half-life, estimated at about 12 minutes (21), and the ability to increase in response to many stimuli (35). Exposure of rats to cold (6,50), their immobilization for short periods of time (50), or their injection with reserpine (50,55) all bring about an induction of the enzyme in the adrenal medulla transynaptically, the effect being mediated through the formation of cyclic AMP (8). There is evidence also of a central cholinergic mechanism regulating the induction of medullary ODC (55,49). The activity of the adrenocortical enzyme can also be stimulated by these stressors (6,13,50), as well as by the phosphodiesterase inhibitor aminophylline (7) and the cholinergic drug oxotremorine (49). In the last case, the cerebral system that includes cholinergic fibers regulates adrenocortical ODC activity indirectly through its influence over pituitary secretion (49). The effect of anterior pituitary hormones on the adrenal ODC activity has been measured in hypophysectomized rats, but thus far without separation of the two parts of the gland. The ODC activity, as measured in such homogenates of whole adrenal, is sensitive to ACTH (28,29,51), growth hormone (28) and prolactin (52,61).

Studies in this laboratory have been directed toward the control mechanisms that regulate the activity of adrenal enzymes. Another inducible enzyme of the adrenal medulla, tyrosine hydroxylase (EC 1.14.16.2)

responds to repeated treatment of rats with dopaminergic agents (15,45,46). Like adrenomedullary ODC (4) it is almost entirely dependent upon intact innervation for induction (46). Because of these similarities it was considered worthwhile to employ two dopaminergic drugs, apomorphine (APM) and piribedil (PBD), to explore the neural pathways involved in the control of adrenal ODC activity. Ordinarily, the specificity of action of these drugs on adrenal ODC activity would be tested with haloperidol HLP), a dopamine-receptor antagonist. Because this drug itself induces adrenal ODC activity (3), it became of interest to determine the conditions under which dopaminergic agonists and HLP might display pharmacological antagonism in relation to this enzymic induction.

Finally, the differential changes in ODC activity caused by the different drug treatment have been examined separately in both adrenal medulla and cortex, as in our previous work (3,49,50); and the kinetic constants for the substrate and the cofactor have been determined in both control and APM-stimulated enzyme preparations.

MATERIALS AND METHODS

Drugs

Piribedil was a gift of Laboratoires Servier, Neuilly-sur-Seine, France; and haloperidol, McNeil Laboratories (Canada) Ltd., Don Mills, Ontario. Apomorphine was purchased from F.E. Cornell and Co., Montreal, Quebec. L-(1-^{14}C)Ornithine, specific activity 40-60 Ci/mol, and 2,5-diphenyloxazole (PPO) were purchased from New England Nuclear, Boston MA. Corticosterone, epinephrine, norepinephrine and epinine were obtained

from Sigma Chemicals, St. Louis MO. All other chemicals were obtained from standard commercial sources.

Animals

Male Sprague-Dawley rats, weighing an average of 200 g, were used throughout this work. Animals were purchased from Canadian Breeding Farms and Laboratories Ltd., St. Constant, Quebec. Hypophysectomized rats were obtained by the same supplier one day after surgery. Animals were kept in the animal room for 4 days after arrival. They were maintained in individual wire cages under a light-dark cycle of 12:12 h with tap water and Purina Checkers ad libitum.

Apomorphine.HCl was dissolved in 0.1% solution of sodium metabisulfite to prevent oxidation of the drug. HLP was dissolved in a few drops of acetic acid and then diluted with distilled water. PBD was suspended in 1% methyl cellulose. All drugs were titrated with 0.1 N NaOH to around pH 6 and injected in a volume of 3 ml/kg body weight. Controls were injected with the same volume of carrier solution.

Tissue preparation

Adrenals were quickly removed from the decapitated rats, weighed, and dissected into medullary and cortical tissue at 4°C with the aid of a magnifying lamp. Contamination of cortical tissue by the medulla was estimated to be about 10%; this was based upon the measurement of free catecholamines present in both dissected portions by ion-pairing high performance reverse phase liquid chromatography (HPLC) with amperometric detection (Table I). The samples were prepared according to the method of

Keller et al. (25) and the conditions for the mobile phase were based on those of Moyer and his colleagues (36), with modifications by J.-P. Gagner of this laboratory (personal communication, see details below). Corticosterone was also measured in both adrenal portions by a microfluorometric method (17) (see details below). Of the total amount of the steroid found in adrenal glands 15% was in the dissected medullary tissue (Table I). As corticosterone is produced mainly in the two inner layers of cells of the cortex (zonae reticularis and fasciculata) (30), and as the contamination by these two layers is expected to be higher than by the zona glomerulosa, which is further away from the medulla, the real degree of tissue contamination would be somewhat less than indicated by steroid analysis.

The portion of the tissue corresponding to two medullae or two cortices was pooled and homogenized with a motor-driven Teflon pestle in 0.2 ml of sodium-potassium phosphate buffer, 0.05 M, pH 6.8. The homogenate was centrifuged at 20,000 g for 20 min. An aliquot of the supernatant (0.1 ml) was used for assay of ODC activity.

In some experiments the supernatant was dialyzed for 18-24 h against 300 volumes 0.05 M phosphate buffer containing 0.05 mM pyridoxal-5'-phosphate, 1.0 mM EDTA, and 0.1 mM dithiothreitol at 4°. The dialysis membrane had a pore size of 4.8 nm, which corresponds to a molecular weight of 12,000 daltons for a spherical molecule. In another group of experiments the supernatant fractions were filtered through Sephadex G-50 to resolve

the enzyme preparations of their cofactor.

Catecholamine determination

The tissue corresponding to one adrenal medulla or one adrenal cortex was homogenized in 0.5 ml of 0.15 M NaCl. Aliquots of 0.05 ml of homogenate were mixed with 0.2 ml of a 0.1 M perchloric acid solution containing 300 ng of epinine, 3.84 µg sodium metabisulfite, and 175 µg EDTA. These mixtures were centrifuged at 12,800 g for 30 min. The deproteinized supernatants were added to an Eppendorf tube containing 15 mg of acid-washed alumina (Woelm, neutral activity, grade I; ICN Canada, Montreal, Quebec), previously equilibrated with 1 ml of 0.5 M Tris-HCl buffer pH 8.4 and 5.9 mM EDTA. After mixing for 15 min, the supernatant was removed by aspiration, and the alumina was washed twice with distilled water. Catecholamines were then eluted from the alumina with 0.1 ml of 0.1 M perchloric acid and 0.1 mM sodium metabisulfite. The eluate was stored at -70° until catecholamines were analyzed. Small samples of 10-30 µl were injected into the HPLC system.

The standards, norepinephrine (NE) and epinephrine (E), and the internal standard epinine were used. Double-distilled water was used to prepare the solvent system, which consisted of: 95.5% of 0.1 M monobasic sodium phosphate; 0.1 mM EDTA and 0.2 mM sodium octyl sulfate adjusted to pH 5.5 with 2 N NaOH; and 4.5% methanol. The HPLC system was composed of a model 6000A solvent delivery system, a U6K model injector and a µBondapak C_{18} column (Waters Scientific Ltd., Mississauga, Ontario). A

model LC-4 electrochemical controller was used with a TL-4 oil-impregnated carbon paste electrode (Bioanalytical Systems Inc., West Lafayette IN). The potential was set at +0.7V versus an Ag/AgCl reference electrode. The solvent system was delivered at a flow rate of 2.5 ml/min. The catecholamines were eluted in the order NE, E and epinine within 12 min; and their concentrations were determined from the peak-height ratios of NE and E over the internal standard epinine.

Corticosterone determination

Each adrenal medulla or cortex was homogenized in 0.5 ml of 0.15 M NaCl in 20% ethanol. Aliquots of 0.05 ml, diluted to 0.2 ml with water, were washed with 3 volumes of iso-octane by shaking vigorously for 30 sec in a conical glass-stopped tube. After centrifugation at 2000 rpm the solvent layer was aspirated. Corticosterone was next extracted into 6 volumes of chloroform. After centrifugation the supernatant was discarded, 0.05 ml of 0.1 N NaOH was added to remove estrogens, and the tube was shaken for 15 sec. The sample was centrifuged again, and the alkali removed by aspiration. An aliquot of 0.750 ml of the chloroform solution was transferred to another tube containing 0.350 ml of ethanol-sulfuric acid reagent (35:65), and the tubes were shaken and centrifuged. The top layer was discarded, and the sample was left to stand at room temperature 45 min. The fluorescence of the sample was determined with excitation at 462 nm and emission at 518 nm. An Aminco-Bowman spectrofluorometer (American Instrument Co. Inc., Silver Spring MD), with slit arrangement No. 2, was used for the analysis.

Ornithine decarboxylase assay

Determination of ODC activity by incubation of the tissue supernatant, obtained by centrifugation at 20,000 g, with L-(^{14}COOH)ornithine and the measurement of radioactive CO_2 liberated was performed by combining elements of the assays described by Russell and Snyder (56) and Jänne and Williams-Ashman (22), with some modification. The reaction mixture contained 1 µCi of L-(-^{14}C)ornithine, 0.04 mM; pyridoxal-5'-phosphate, 0.05 mM; dithiothreitol, 1.0 mM; EDTA, 0.1 mM; phosphate buffer, pH 6.8, 50 mM; and 100 µl of the enzyme preparation in a total volume of 0.5 ml. The incubation was carried out in a 25 ml conical flask, equipped with a rubber stopper from which a polypropylene well was suspended (Kontes Glass Co.). The enzyme preparation was preincubated for 10 min at 37° in a shaking water bath. The reaction was begun by the addition of the substrate. After 45 min incubation the reaction was stopped by the addition of 0.5 ml of 5 N sulfuric acid. The $^{14}CO_2$ produced during the reaction was trapped in a 2 cm^2 filter paper (Whatman No. 3) impregnated with 100 µl of a mixture of ethyleneglycol monomethyl ether and monoethanolamine (2:1) and contained in the well. The flasks were shaken for an additional hour to insure trapping of all $^{14}CO_2$ released.

At the end of the incubation, the filter paper was transferred to a vial containing 10 ml of a mixture of toluene, ethyleneglycol monomethyl ether (2:1) containing 0.4% PPO for liquid scintillation counting in a Beckman spectrometer.

The assays for the determination of the cofactor kinetic constants were carried out in the dark to prevent significant oxidation of low concentrations of pyridoxal-5'-phosphate.

Statistical procedures

Data in the tables are presented as mean ± standard error of the mean (SEM). As the latter value tended to vary directly with the size of the mean, individual values were transformed logarithmically for calculation of statistics used in assessing the significance of differences, i.e. Student's t in comparing two groups and Fisher's F in the analysis of variance (57).

RESULTS

Diurnal variation and effect of injection

The lower curves in Figure 1 for adrenal cortex and medulla are derived from an experiment in which groups of 4 rats maintained in quiet surroundings were killed at various times during the day for measurement of ODC activity. The other curves in Figure 1 represent data compiled from control groups of animals used in many experiments, and given different schedules of injection with inert vehicle. As can be seen, one or more control injections were sufficient to double or triple cortical and medullary ODC activity. Despite the wide distribution of mean values, individual determinations exceeding 100 and 50 pmol per mg protein per 45 min incubation, for adrenal cortical and medullary ODC activity, respectively, were seldom encountered among the control rats.

In view of this response to the stress of handling and injection, care was always taken that each experimental group had its own matching controls, treated under the same conditions. To avoid any effect of diurnal variation the rats were killed around the same time of day in the different experiments.

Effects of piribedil and apomorphine on adrenal ODC activity

A single intraperitoneal injection of PBD (50 mg/kg) was followed by an increase in the activity of adrenal ODC in both medulla and cortex (Figure 2). The adrenal medulla responded faster; that is, there was a significant increase ($P < 0.001$) at 2 h. Cortical ODC did not attain a significant increase until 4 h after administration of the drug. This point corresponds to the maxima for both adrenal medulla and cortex. Thereafter, the activity of the enzyme decreased in the medulla, but remained elevated for at least 8 h in the cortex. Fourteen hours after the administration of PBD, the ODC activity had returned to basal levels.

APM (10 mg/kg) was injected intraperitoneally, and ODC activity of the adrenals was measured at various times afterwards, as in the case of the PBD experiments. There was no increase of the enzymic activity. When a second dose was given 1.5 h after the first, both medullary and cortical ODC activities increased. The time-course of stimulation showed that there were increases in the adrenal at 2 h after the first APM injection (Figure 2), the increase being significant ($P < 0.01$) in the case of the medulla. At 4 h after the initial injection ODC activity was high in both structures, and was still rising in the cortex at 6 h. Thus,

similar patterns of induction of adrenomedullary and cortical ODC activity are provoked by the administration of two different dopaminergic drugs: APM (2 doses of 10 mg/kg, with an interval of 1.5 h between them) and PBD (single dose of 50 mg/kg).

Effect of haloperidol on apomorphine induction of adrenal ODC activity

To study the effect of HLP, rats were given this drug in a dose of 5 mg/kg intraperitoneally, followed 0.5, 2.0 and 3.5 h later by injection of APM, 10 mg/kg on each occasion. Controls, which received HLP and subsequent injections of vehicle, showed small, but not statistically significant, increases of adrenal ODC activity at 4.5 h, but by 6.5 h HLP-treated rats had large increases in the enzymic activity in both adrenal medulla and cortex (Figure 3).

With the injection schedule described for APM (3 doses of 10 mg/kg i.p.) the drug induced ODC activity as before, but the prior administration of HLP blocked the effect of APM acting for 4 h in both adrenal cortex and medulla. When this protocol was followed once more, but with measurements of the ODC activity at 6.5 h after the injection of HLP alone, there were large increases in both medullary and cortical ODC activity; these were similar to those elicited by APM itself. At this time, the combination of the drugs had no further effect on adrenal ODC of the animals than did the administration of either drug alone. In fact, the mean effect of the combination was less than that of HLP alone, for both tissues, but these differences were not statistically significant (Figure 3).

In other experiments HLP was tested for blocking action against PBD. The latter drug was injected 0.5 h after the HLP, and ODC activity of the

adrenal glands was measured 2 h later. The results (Table II) show that HLP and PBD individually provided significant increases in medullary ODC activity. However, their combination gave much less of an increase than expected for additive effects. Indeed, the results suggest antagonism by HLP of the action of PBD. At this time there were no significant effects on cortical ODC.

Effect of hypophysectomy

In order to determine if the anterior pituitary gland exerts any influence on the mediation of these effects, the next experiment was performed with hypophysectomized rats. These were given the same drug treatments as just described, and the adrenals were taken for dissection 6 h after the first of three APM injections. The results in Figure 3, right side, show that hypophysectomy practically eliminated the increase in ODC activity in the medulla that has been obtained with HLP in intact rats, and considerably reduced the increase induced by treatment with APM. Combining the two drugs resulted in a partial antagonism.

As for the cortex, the rise of ODC activity seen in intact animals given either APM or HLP, or both, was prevented by hypophysectomy. The small increase in cortical ODC activity after APM can be attributed to contamination with medullary tissue (see Methods). Thus, the effect of HLP by itself was wholly dependent upon the presence of the pituitary gland. This result suggests that the increases in enzymic activity of the medullary tissue of intact rats at 6 h could have been partly due to the presence of cortical tissue embedded in it and not removed in the dissection (see Tissue preparation).

Effect of apomorphine administration on the kinetic characteristics of ornithine decarboxylase

The changes observed in ODC activity after the administration of APM can result from an alteration in catalytic activity of pre-existing enzyme, an increased content of enzyme or both. Therefore, to study the mechanism of the altered enzyme level the kinetic constants of ODC of the adrenal medulla and cortex in control and APM-treated rats were determined. To measure the kinetic constants for the substrate, the enzyme extracts were dialyzed in the presence of added cofactor as described in Materials and Methods. As for the cofactor, the enzyme extracts were filtered through Sephadex G-50 in the absence of pyridoxal-5'-phosphate (PLP). Dialysis and gel filtration each exclude small molecular-weight molecules, up to 12,000 and 30,000 daltons, respectively.

Dialysis of the samples enhanced the activity of ODC 1.5 to 3 times in the medulla and cortex of control and APM-treated rats. However, gel filtration did not increase the specific activity of the preparations; in fact, there was variable loss of enzyme in different preparations ranging from 45 to 10%. The crude undialyzed preparations of medullary and cortical ODC had about 15% activity without added PLP. Upon filtration through Sephadex, the activity was practically undetectable without added cofactor. Storage of such preparations at -80^{o} entailed loss of 10% of the activity after 12-16 h and about 60% in one week.

Mixing experiments were carried out in undialyzed, dialyzed and gel-filtered enzyme preparations to determine whether the changes in ODC

activity after the drug treatment were due to the presence of an activator or loss of an inhibitor. Studies with mixtures of dialyzed and gel-filtered ODC preparations from control and APM-stimulated rats showed that the activity was always additive (Table III). This was not the case for undialyzed preparations of the enzyme where the activity of the 1:1 enzyme mixture displayed activity lower than the calculated value.

The apparent K_m values for L-ornithine evaluated by least-squares fit of points in a double-reciprocal plot of V versus (S), averaged 0.1 mM and 0.07 mM for preparations of control and APM-treated rats, respectively. ODC in adrenal medulla and cortex had identical K_m values but different V_{max}. The administration of APM, in this manner, produced no significant change in the constant of affinity of ODC for its substrate. However, the apparent V_{max} was increased 7-10 fold for the medulla and 3-4 fold for the cortical enzyme (Figure 4, Table IV).

As can be seen in Figures 5,6 and 7, non-linear, Lineweaver-Burk plots of ODC activity as a function of PLP concentration were produced. From these plots two different K_m values were calculated by least-squares fit in enzyme preparations from whole glands, or adrenal cortex of control or APM-treated, or from adrenal medulla of APM-treated rats. In addition, the V_{max} was increased considerably (3-10 fold) after the administration of APM in all cases. The absolute values for the V_{max} cannot be compared because of the variable loss of activity of the different ODC preparations passed through Sephadex (Table IV).

Effect of cycloheximide on the increase in adrenal ODC activity after apomorphine administration

To determine whether the increases in ODC activity in adrenal medulla and cortex are due to protein synthesis de novo, the inhibitor cycloheximide was administered to rats 30 min prior to APM. These data, presented in Figure 8, showed that cycloheximide prevented the large increase in ODC activity normally seen after APM administration in adrenomedullary and cortical tissues. These results then suggested that APM-induced increase in ODC activity is mainly due to an increase in the amount of protein.

DISCUSSION

Effect of the drug treatments on adrenal ODC activity

In this work three drugs acting upon dopamine-sensitive receptors have been studied in relation to the activity of adrenal ODC activity. APM is considered to be a very specific dopaminergic agonist acting by mimicking the effect of dopamine at receptor sites in the central nervous system. Both APM and PBD, another dopamine agonist, reduce the turnover of neostriatal dopamine (5,11). This effect has been explained in terms of feedback inhibition of dopaminergic function caused by the direct stimulation of presynaptic dopamine receptors. Both drugs cause increased locomotor activity as well as stereotyped behavior, consisting of repetitive sniffing, licking, chewing and agitation. This stereotyped behavior is dose-related in intensity (32) and duration (26).

In regard to ODC, its induction in the adrenal medulla under the influence of APM is centrally mediated, as deduced from experiments with unilaterally splanchnicotomized animals (4). In intact rats a single dose of APM (10 mg/kg) did not have an effect on the activity of that enzyme in either the adrenal medulla or cortex up to 4 h after its administration. At least one additional injection of the drug (at an interval of 1.5 h in our work) was necessary to increase ODC activity. Similar results have been obtained with adrenal tyrosine hydroxylase (45,46), although the time-relation of doses of drug in that case is quite different. A single dose of PBD was sufficient to raise ODC enzyme activity in both structures. The enzyme activity responded faster in the medulla after the administration of either APM or PBD than in the cortex, a fact that has been observed also in exposure of rats to cold (6), immobilization (50), or administration of either oxotremorine (49), or HLP (3). The fact that a single dose of APM, 10 mg/kg, did not increase adrenal ODC activity, whereas a dose of PBD, 50 mg/kg, did so is probably explained by the need for the drug to act effectively for a certain period of time during which the inductive mechanism is called into play. These factors are determined by the rate of metabolism of the respective drugs. Costall and Naylor (12) reported that the maximal intensity of stereotypy induced by APM, 2 mg/kg, occurs at 15 min, and that caused by PBD, 50 mg/kg, at 2 h after their respective administration. Moreover, the intensity of the stereotyped activity was greater for APM than PBD under the conditions of their experiment. Lal and Sourkes (26) observed

a rapid onset of stereotypy after a dose of 10 mg/kg APM, given i.p., the behavior lasting for about 68 min. It has been shown that with the dose of APM used in this work the concentration of the drug in the brain reaches a peak at 5 min after its injection, and then declines rapidly, with only trace amounts remaining at 1.5 h (58).

HLP, a typical neuroleptic and dopamine antagonist (23), blocks the action of dopamine at postsynaptic receptor sites and counteracts the effects of APM (37). In this work HLP had at least two discernible effects. The first was the antagonism of the APM-induced increase in adrenal ODC activity. This can be explained by competition at the receptor site in the CNS, but another factor may have a role: it has recently been reported that pretreatment of rats with HLP prevents the accumulation of APM in the striatum in a dose dependent fashion and in parallel to a reduction in the animals' gnawing behavior (62). HLP also reduces the locomotor activity stimulated by APM administration (38). The antagonism of the APM-induced increase of ODC activity by HLP was observed in our particular experiments with intact rats at 4.5 h after the HLP injection, and at 6.5 h in hypophysectomized animals (Figure 3). The second effect of HLP was a stimulatory one on ODC activity, observed in intact rats, in this case at 6.5 h after HLP administration.

These results must be examined in the light of evidence that cortical ODC responses are strongly influenced by hypothalamo-pituitary factors, whereas medullary ODC activity seems to be more susceptible to changes in sympathetic activity (50). In regard to the effect of HLP on cortical ODC

activity (as shown in Figure 3), it is known that this neuroleptic has a depressing effect upon the secretion of prolactin-inhibitory factor of the hypothalamus, with a consequent action in increasing the serum concentration of prolactin (14). HLP administration also leads to an increase in plasma ACTH (16). In other words HLP acts, presumably in the hypothalamus, to cause increased release of two pituitary hormones into the serum, both of which favor the induction of (whole) adrenal ODC activity. This may be a sufficient explanation for that effect of HLP. However, such a mechanism would no longer operate in hypophysectomized rats, and the result obtained with HLP for both adrenal medullary and cortical ODC in such animals corresponds precisely to this fact, as seen in Figure 3. Thus, the increase caused by HLP 6.5 h after its administration can be attributed entirely to mediation by the hypophysis. Local effects of APM and HLP on adrenal tissue have already been excluded through failure of these drugs, incubated with whole rat adrenals or slices of beef adrenal gland, to cause any increase of ODC activity (results not included). Moreover, the antagonistic action of HLP towards APM in regard to induction of adrenal medullary ODC in hypophysectomized rats (Figure 3) must be considered as taking place at some central site independent of the hypothalamo-hypophyseal system. Further research in our laboratory is aimed at elucidating the central dopaminergic pathways involved in the regulation of adrenal ODC activity.

Effect of apomorphine in the kinetic characteristics of ODC

Kinetic studies of dialyzed adrenomedullary and cortical ODC preparations

from control and APM-treated animals showed that there was no appreciable change in the K_m of the enzyme for the substrate L-ornithine. Moreover, a pronounced increase in the V_{max} in medulla and cortex of APM-treated rats was observed, as had been previously found for exposure of animals to cold or oxotremorine (48). The average K_m value in control and APM-treated rats for both portions of the adrenal was 0.09 mM; this value is similar to that reported in the literature for thioacetamide-treated and regenerating rat liver (39,41,47), rat prostate (2,18,22), and Swiss 3T3 mouse fibroblasts (10).

When dialyzed preparations from adrenals of control and APM-treated rats (whole gland or separated medulla and cortex) were incubated together and then assayed, the enzyme activities were additive. This indicated that the increase in activity was probably due to an increase in the amount of the enzyme and not to the presence of an activator or the loss of an inhibitor. Furthermore, the induction was prevented by pretreatment of the animals with cycloheximide in both the adrenal medulla and cortex. Earlier it has been shown that protein synthesis de novo is involved in the increase in ODC activity of adrenals of rats exposed to cold or treated with aminophylline (7) or ACTH (51). These increases in ODC activity are also dependent upon RNA synthesis, because treatment of rats with actinomycin D blocks the increase (7,29).

Immunologic techniques would provide a more definitive proof that the altered levels in ODC activity represent an increase in protein content.

Although antibodies to ODC have been reported by several laboratories (19,24,40,60,63) the enzyme is quite unstable during purification (9,31, 43). Hence, most studies have been limited to measurements of ODC activity. Our own results have shown that much is lost just by storing ODC in the absence of cofactor or by passing the enzyme extract through Sephadex. The second and more-limiting factor for the purification of ODC in adrenal is its very low specific activity.

The 1.5-3-fold increase in activity observed after dialysis of adrenal ODC preparations suggests that a small molecular weight inhibitor(s) is removed. The work of Ramirez-Gonzalez (48) showed that nondialyzed enzyme preparations of adrenal medulla and cortex have an apparent K_m of 0.22 and 0.09 mM, respectively. After dialysis, a K_m of 0.05 was obtained for both medulla and cortex. Similarly, Pegg and Williams-Ashman have observed an increase in rat prostate ODC after dialysis (42). The failure by Abdel-Monem et al (1) to detect any increase in the same tissue may be due to the fact that they did not add PLP to the dialysis buffer, as dialysis of ODC preparations from Swiss 3T3 mouse fibroblasts and rat prostate, in the absence of PLP, reduced the activity of the enzyme (10,42). The product of the reaction putrescine, and the polyamines for which it is precursor, are good candidates for the inhibition of ODC in the adrenal; they inhibit ODC in regenerating rat liver (20,24,44) and in many cell lines and systems. Putrescine binds covalently to ODC in the presence of transglutaminase in purified preparations of thioacetamide-stimulated calf liver (54). This transamidation of putrescine to ODC results in a

linear decrease in activity.

Kinetic studies to determine the affinity constant for the cofactor have shown that ODC in whole adrenal gland of control rats has two different apparent K_ms for PLP. Adrenal tissue from rats treated with APM show similar results. In order to find whether only one of the two enzymes forms is present in each part of the adrenals, the glands were dissected into medullary and cortical tissues as before. The adrenal medulla of control, at the substrate concentration studied, showed only one K_m value. The ODC of adrenal cortex in controls, on the other hand, had two apparent K_ms. In APM-treated rats, ODC of the cortex and medulla had two apparent K_ms each.

Thus, it is possible that each part of the adrenal gland contains two enzyme forms, rather than one form of the enzyme per specific part of the gland. Two different enzyme forms have been purified in Physarum polycephalum differing in their affinity for the cofactor (33), and in crude preparations of rat prostate (2) and Swiss 3T3 mouse fibroblasts (10). These forms, which have very different affinities for PLP, have similar, if not identical, affinities for ornithine.

Both the activity and stability of ODC in the adrenal gland are strongly dependent on PLP in our dialyzed enzyme preparations, as previously seen in undialyzed preparations (48), and in prostatic ODC (1,2,42).

Finally, the existence of two ODC forms in the adrenal gland of the rats can be an alternative mechanism for the regulation of its activity as has been suggested for Physarum (33,34), developing rat heart (27), rat

thymus and kidney (53) and rat liver (39,40).

ACKNOWLEDGEMENTS

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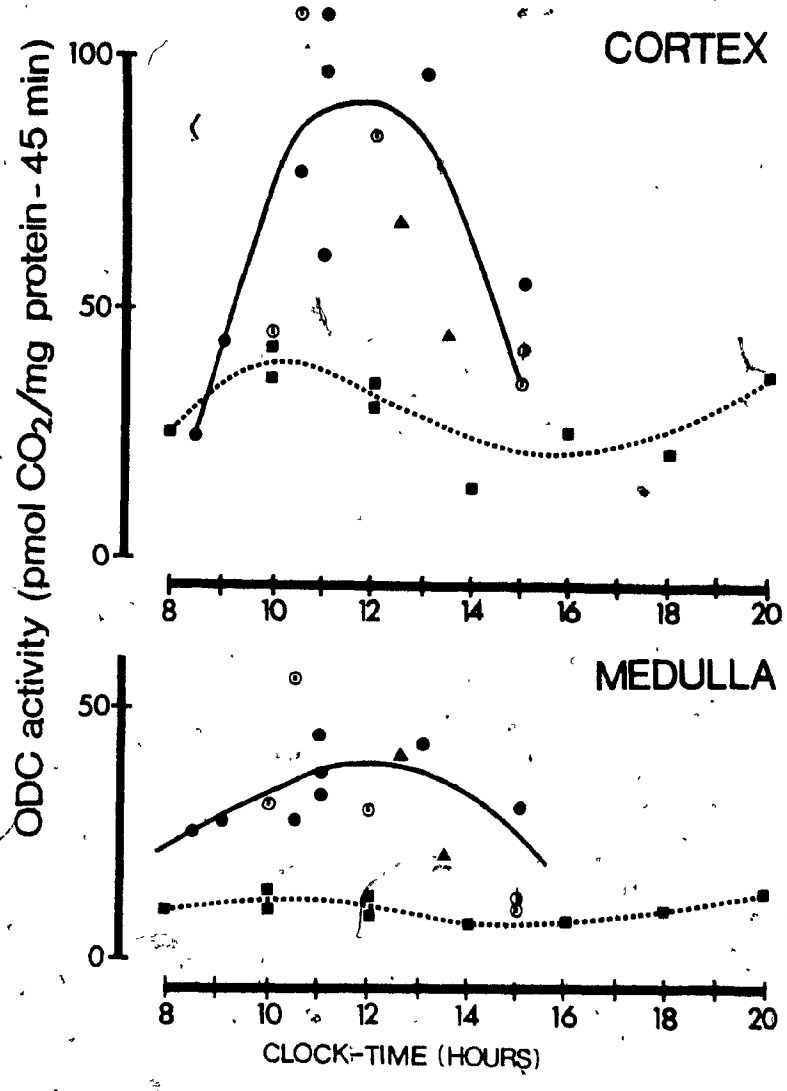

FIGURE 1

Diurnal variation and the effect of injection. The symbols represent mean values for 4 to 6 rats receiving 1 to 4 injections of vehicle (control animals) at scheduled times during the course of various experiments. As there was no systematic variation of ODC activity in relation to the number of injections or their timing, this information has not been included in graph.
○, one injection; ⊙, two injections; ◉, three injections; ▲, four injections.
The lower curves for each of the two parts of the adrenal glands are derived from one experiment in which animals were not stressed by handling or injection ■, (no injection).

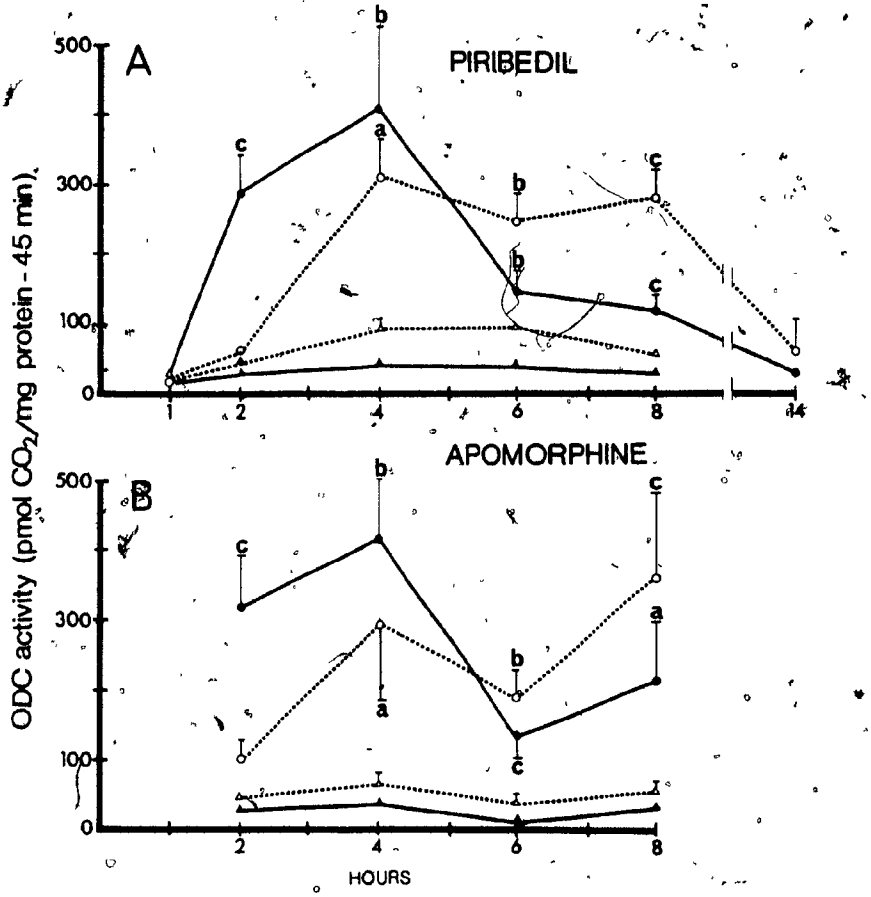

FIGURE 2

Time-course of stimulation of adrenal ODC activity after the administration of dopaminergic agents. A. Piribedil, 50 mg/kg i.p. given at 0 time. B. Apomorphine, 10 mg/kg i.p. given at 0 and 1.5 h. The following symbols were used: ●, medulla, drug-treated animals; ○, cortex, drug-treated; ▲, medulla, controls; △, cortex, controls. Values are presented as mean ± SEM for 406 rats. Significance of differences from control means: $^{a}p < 0.05$; $^{b}p < 0.01$; $^{c}p < 0.001$.

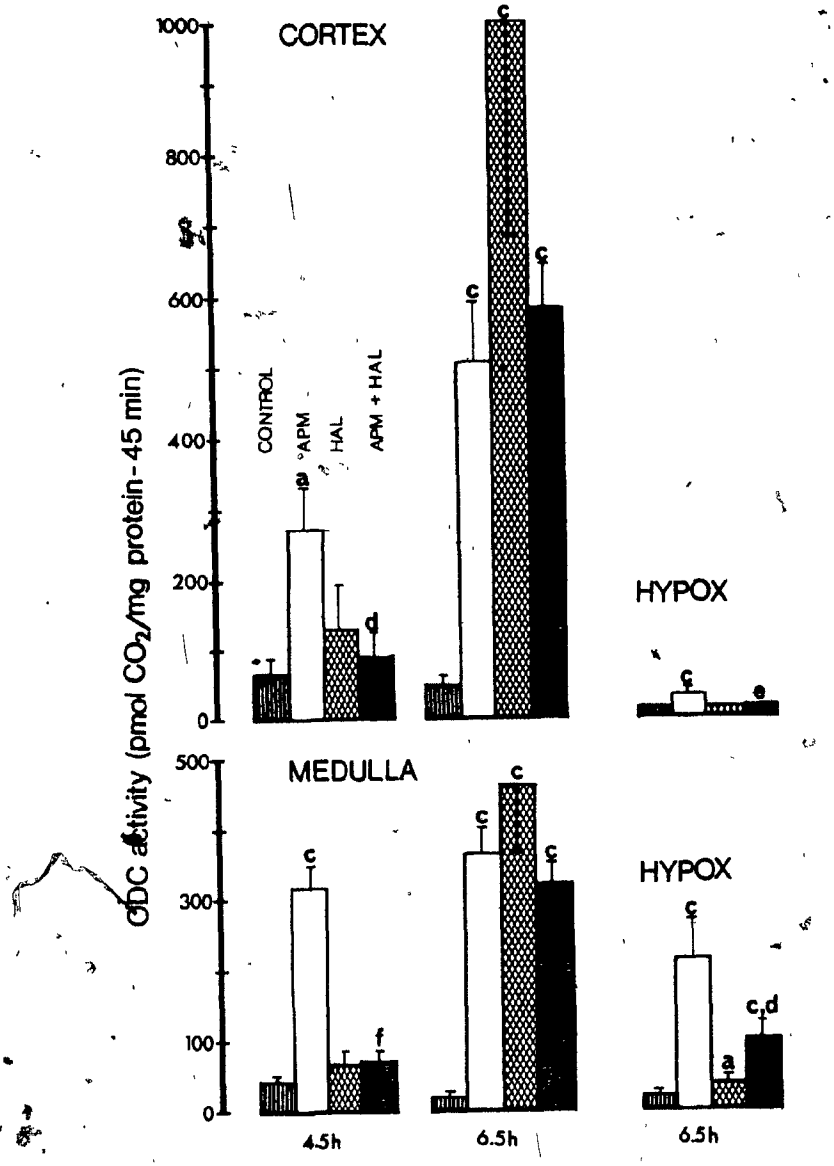

FIGURE 3

Effects of apomorphine (APM) and haloperidol (HLP) given alone and combined on ODC activity of the adrenal gland. APM was injected in 3 doses of 10 mg/kg i.p. at 0, 1.5 and 3 h. HLP, 5 mg/kg i.p. was given as a single dose 0.5 h before the first dose of APM. Rats were decapitated at 4 or 6 h after the initial dose of APM (i.e. 4.5 h and 6.5 h, respectively, after injection of HLP) in intact rats, and at 6 h in hypophysectomized rats. Significance of differences from control means: $^{a}p < 0.05$; $^{b}p < 0.01$; $^{c}p < 0.001$. Significance of difference from mean of APM-treated rats: $^{d}p < 0.05$; $^{e}p < 0.01$; $^{f}p < 0.001$.

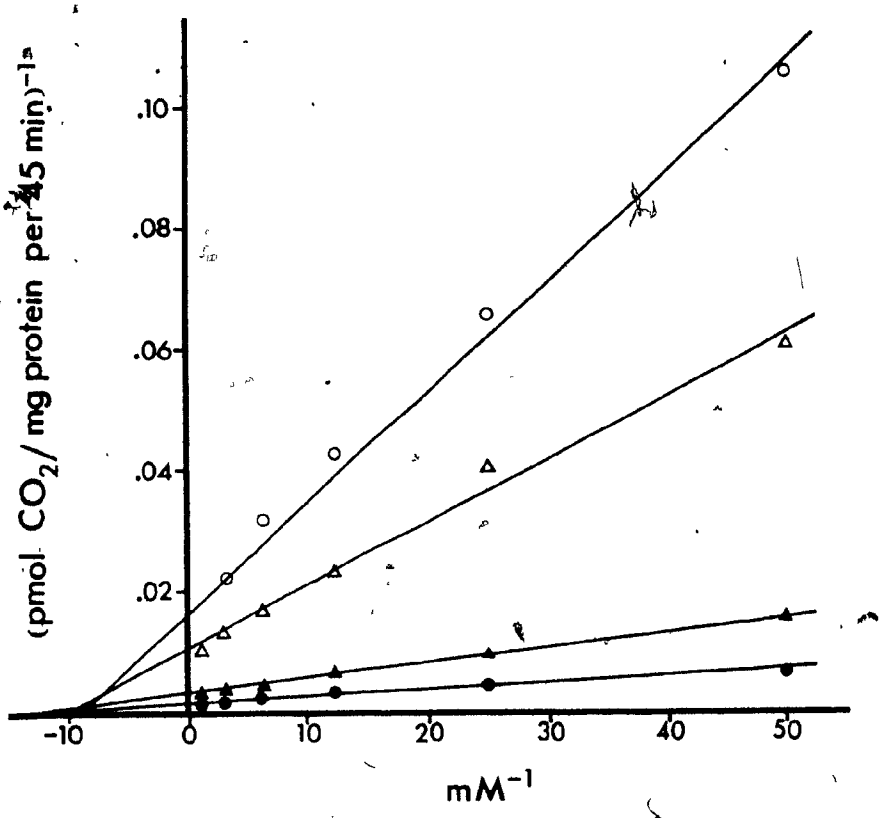

FIGURE 4

Double reciprocal plots of dialyzed ODC as a function of L-ornithine concentration. The enzyme was assayed as described under Methods, in preparations from control adrenal medulla (O) and cortex (Δ), and from APM-treated medulla (●) and cortex (▲).

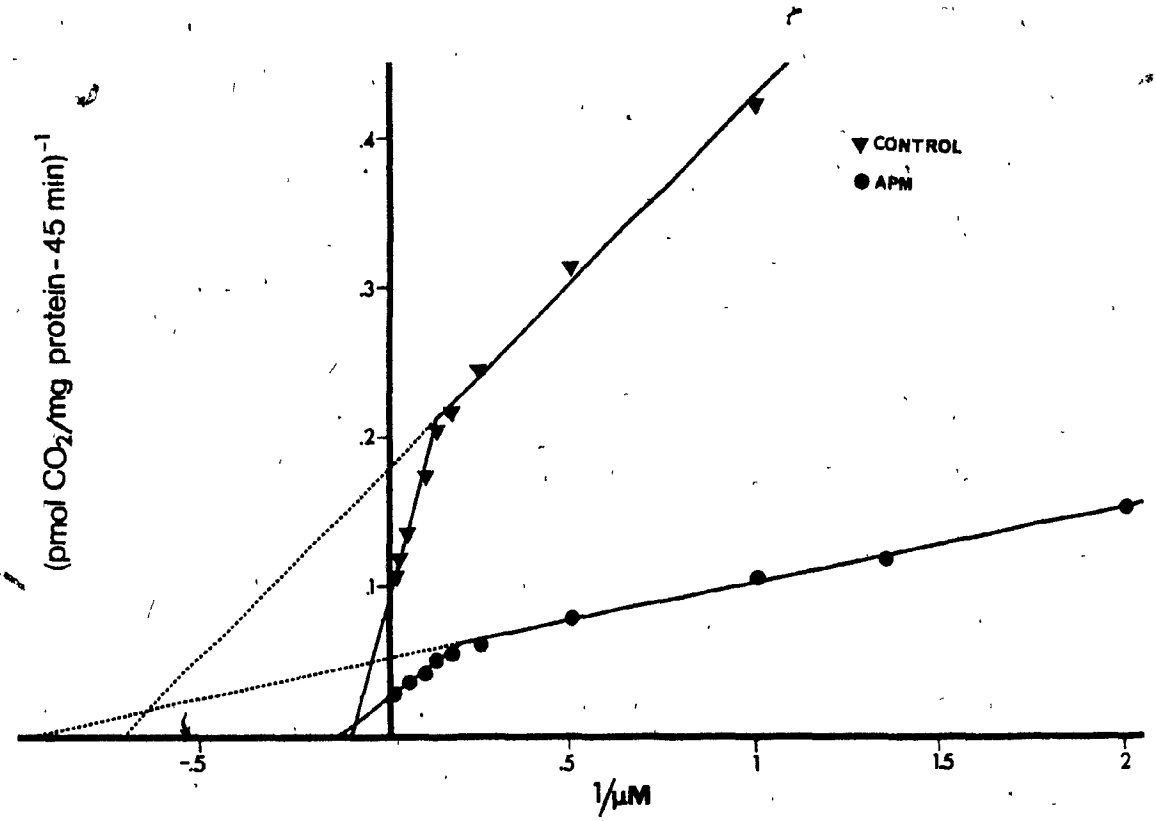

FIGURE 5

Double reciprocal plots of gel filtered adrenal ODC as a function of pyridoxal 5'-phosphate concentration. The enzyme was assayed at a L-ornithine concentration of 0.04 mM.

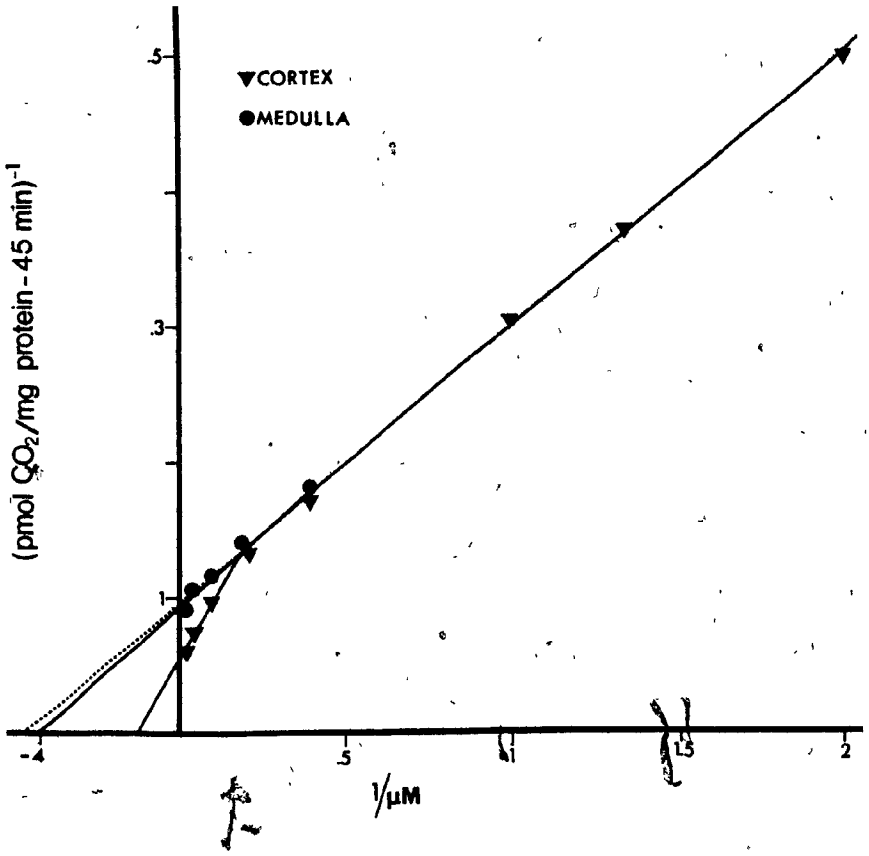

FIGURE 6

Double reciprocal plots of gel filtered adrenomedullary and cortical ODC of control rats as a function of pyridoxal 5'-phosphate concentrations. The enzyme was assayed at an ornithine concentration of 0.04 mM.

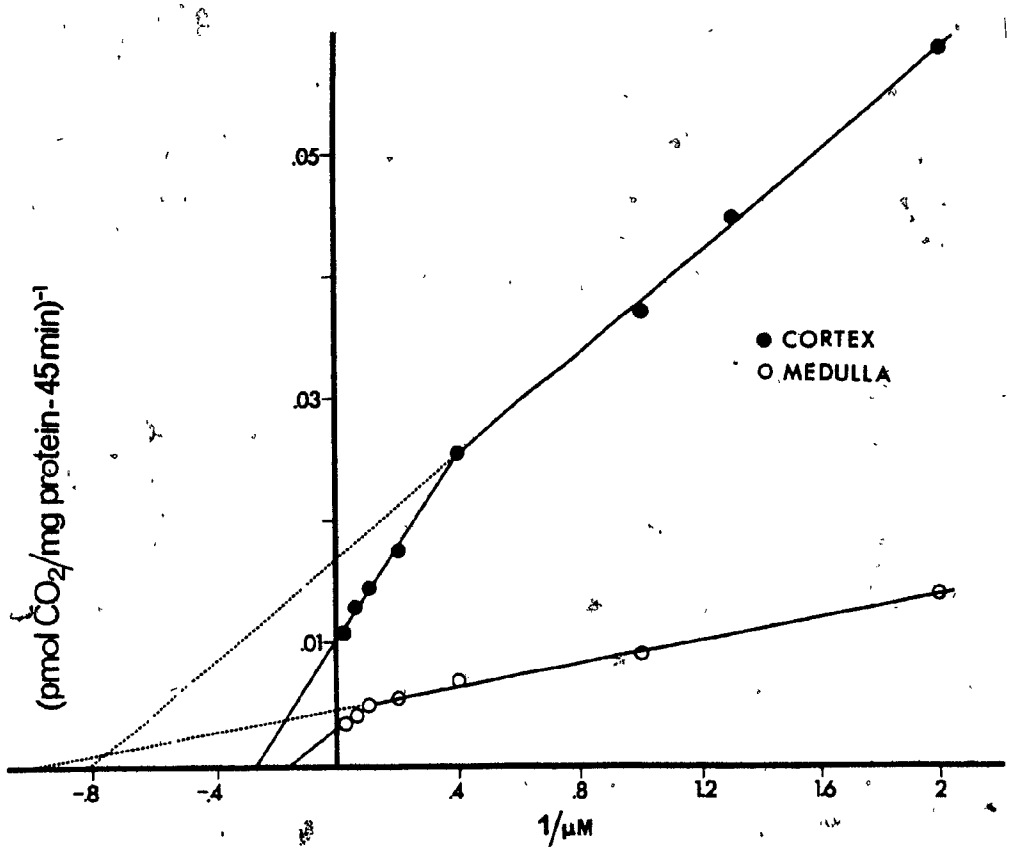

FIGURE 7

Double reciprocal plots of gel filtered adrenomedullary and cortical ODC of APM-treated rats as a function of pyridoxal 5'-phosphate concentrations. The enzyme was assayed at an ornithine concentration of 0.04 mM.

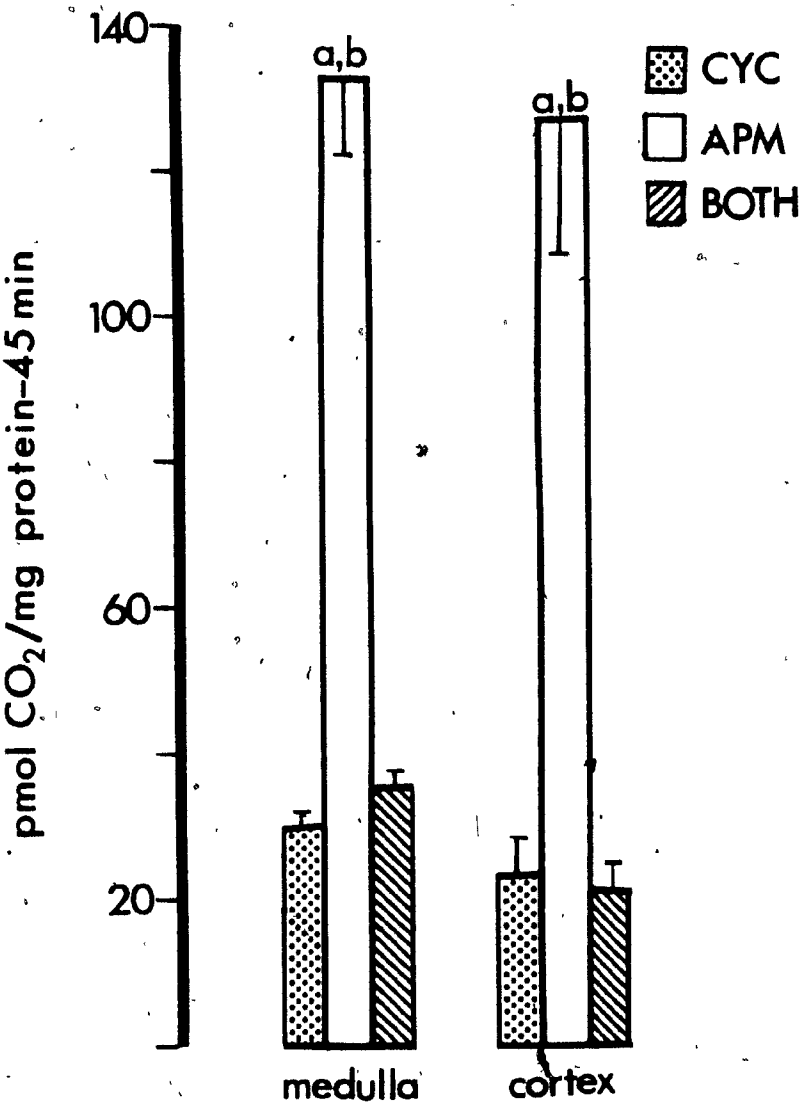

FIGURE 8

Effect of cycloheximide (CYC) on the APM-induced increase in adrenal ODC activity

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CYC (50 mg/kg i.p.) was administered 0.5 h before the first of 3 injections of APM (10 mg/kg i.p. at 0, 1.5 and 3 h). Animals were killed 1 h after the last APM injection. Bars represent the mean ± SEM for 5 rats in each group.

[a] $P < 0.001$ for comparison with CYC-treated
[b] $P < 0.001$ for comparison with (CYC + APM)-treated

TABLE I Concentration of catecholamines and corticosterone in the dissected adrenomedullary and cortical tissue

Parameter	Medulla	% of total	Cortex	% of total
Adrenaline	8.86 ± 0.96	88%	1.22 ± 0.15	12%
Noradrenaline	2.98 ± 0.31	91%	0.28 ± 0.02	9%
Corticosterone	59 ± 7	14%	366 ± 52	86%

Values are expressed as µg of adrenaline, noradrenaline, or ng of corticosterone per adrenal medulla or cortex ± SEM for 10 determinations.

TABLE II Effect of haloperidol on adrenal ODC response to piribedil

Treatment	ODC activity			
	Medulla		Cortex	
Control	56 ± 19.4	(4)	108 ± 39.8	(4)
Haloperidol	161 ± 8.8[b]	(5)	194 ± 49.3	(5)
Piribedil	323 ± 62.5[c]	(6)	63 ± 19.2	(6)
Both	171 ± 62.3[a,d]	(4)	73 ± 23.6	(4)

Values shown represent mean ± SEM (No. of animals). Haloperidol, 5 mg/kg, i.p. was given at 0 time. Piribedil, 50 mg/kg, i.p. was injected at 0.5 h. The animals were killed at 2.5 h. Activity of ODC is expressed as pmoles $^{14}CO_2$ per mg protein in 45 min.

The significance of differences from control means is designated as follows: [a]$P < 0.05$; [b]$p < 0.01$; [c]$P < 0.001$;

[d]Significantly different from piribedil-treated rats, $P < 0.05$.

TABLE III Mixing experiments of ODC preparations from control and apomorphine-treated rats

Animals were injected with APM (3 doses of 10 mg/kg i.p. at 0, 1.5 and 3 h) and were killed 4 h after the first injection. The values represent the mean ± SEM of 4 determinations and are expressed as cpm/sample. For preparation of samples see Material and Methods.

Treatment	Whole gland	Medulla	Cortex
	(cpm)		
	Sephadex G-50	Dialyzed	
APM	1437 ± 15	5089 ± 38	4158 ± 50
Control	393 ± 3	425 ± 5	1226 ± 13
1:1 mixture	917 ± 10	2670 ± 30	2730 ± 36
Calculated values	915 ± 9	2757 ± 21	2692 ± 31
	Non-Sephadex G-50	Undialyzed	
APM	1672 ± 17	1448 ± 16	2310 ± 25
Control	796 ± 8	279 ± 3	816 ± 8
1:1 mixture	930 ± 10	580 ± 7	1295 ± 16
Calculated values	1234 ± 13	863 ± 9	1563 ± 17

TABLE IV Effect of APM on the kinetic characteristics of adrenomedullary and adrenocortical ODC in the rat

Tissue	Treatment	K_m (mM)	V_{max}
		Experiment 1	
Medulla	Control	.11	86
	APM	.07	627
Cortex	Control	.12	96
	APM	.07	273
		Experiment 2	
Medulla	Control	.10	61
	APM	.07	608
Cortex	Control	.10	96
	APM	.08	368

All enzyme preparations were dialyzed as described in Materials and Methods. L-Ornithine concentrations were varied from 0.02 mM to 0.8 mM with a constant pyridoxal 5'phosphate concentration of 0.05 mM. The K_ms and V_{max}s for L-ornithine were determined by the Lineweaver-Burk method at 5-6 substrate concentrations with 2 determinations at each point. In each experiment the K_m was evaluated by the least squares fit.

TABLE V Effect of APM on the kinetic characteristics of ODC in the whole adrenal gland and separated medulla and cortex

Tissue	Treatment	K_m(μM)	V_{max}	% Activity recovered
Whole gland	Control	9.4 1.4	10.8 5.6	75
Whole gland	APM	6.9 1.1	38.1 20.4	55
Medulla	Control	2.4	10.9	85
Medulla	APM	5.8 1.0	330 221	90
Cortex	Control	7.4 2.2	18.2 10.6	80
Cortex	APM	3.6 1.2	95.0 59.3	70

All enzyme preparations were filtered through Sephadex G-50 to remove endogenous pyridoxal 5'-phosphate (PLP). The % activity recovered after this procedure is expressed on the right hand of the table.

PLP concentrations were varied from 50 μM to 0.5 μM with a constant L-ornithine concentration of 0.04 mM. The K_ms and V_{max}s for PLP were determined by the Lineweaver-Burk method at 5-12 cofactor concentrations with two determinations at each point. In all cases, except for the medulla in control animals, two kinetic forms were found; the high-affinity form was evaluated from the 4-6 lowest concentration points and the low affinity form from the 6-4 highest. In each experiment the K_m was evaluated by the least squares fit.

The V_{max} is expressed as pmol $^{14}CO_2$/mg protein per 45 min.

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CHAPTER III

Effect of Haloperidol on Adrenal Ornithine Decarboxylase Activity of the Rat

ABSTRACT

The administration of the dopamine antagonist haloperidol to rats produced a temporary increase in adrenomedullary and cortical ODC activity. The time-course of stimulation of ODC activity by HLP showed different patterns in both structures. Medullary ODC activity was highest at 2.5 h, decreasing at later times; cortical ODC activity was not affected by the drug at 2.5 h, but then increased up to at least 6.5 h. The medullary increase observed at 2.5 h was dose-related and could be prevented by splanchnicotomy. Hypophysectomized rats, on the contrary, showed an enhanced response to HLP. The results suggest that haloperidol-induced increase of adrenomedullary ODC activity is caused by a reflex increase in preganglionic nerve activity, and that the pituitary gland can modulate this response. Cortical ODC response to HLP, as previously demonstrated, is mediated entirely by the hypophysis.

INTRODUCTION

Haloperidol (HLP), a typical neuroleptic and dopamine antagonist (13), has been used in this laboratory to block the action of the two dopaminergic drugs apomorphine and piribedil in stimulating increases of adrenal ornithine decarboxylase (ODC, L-ornithine carboxylase, EC 4.1.1.17) activity. Additionally, it has been observed that HLP itself produced an increase in adrenomedullary ODC activity 2.5 h after its administration (1). Treatment of rats with cholinergic drugs (18,21) or reserpine (9,18,21) also causes an increase in medullary ODC activity. Furthermore, these effects are transynaptically mediated (17,18,21).

To study further the effect of HLP on adrenal ODC activity and its mode of action, the time-course of stimulation and the dose-response relationships have been investigated in separated adrenal medulla and cortex.

MATERIAL AND METHODS

Animals

Male Sprague-Dawley rats (Canadian Breeding Farms and Laboratories Ltd., St. Constant, Quebec), weighing 190 - 210 g, were housed in individual wire cages. They were kept at 25° on a 12 h light-dark schedule with tap water and Purina Checkers ad libitum.

Animals were killed by decapitation between 10 AM and 2 PM to avoid diurnal variations in ODC activity (1).

Hypophysectomy and splanchnicotomy.

Hypophysectomized rats were obtained from the supplier mentioned one day after surgery. Unilateral splanchnicotomy was performed in this laboratory with the aid of a dissecting microscope under chloral hydrate anesthesia (300 mg/kg). The weights of the denervated and innervated adrenals (14.3 ± 0.3 left, 13.6 ± 0.3 right, in mg ± SEM, for n = 45) were almost identical; this lent assurance that the vascular supply to the denervated gland had remained intact. Both hypophysectomized and splanchnicotomized rats were used for experiment four days after the operation.

Drugs

Haloperidol was donated by McNeil Laboratories (Canada) Limited, Don Mills, Ontario. L-(1-^{14}C)Ornithine, specific activity 40-60 Ci/mole, and 2,5-diphenyloxazole (PPO) were purchased from New England Nuclear (Boston, MA), and chloral hydrate from Fisher Scientific Co., Montreal, Quebec. All other chemicals (analytical quality) were obtained from standard commercial sources.

HLP was dissolved in a few drops of glacial acetic acid, diluted with distilled water and then titrated with 0.1 N NaOH to pH 6. The drug solution was injected intraperitoneally in a volume of 3 ml/kg body weight. Controls were injected with the same volume of carrier solution.

Statistical treatment

Data are expressed as mean ± standard error of the mean (SEM). As the SEM varied directly with the size of the mean individual values were transformed logarithmically, before Student's t-test was applied, to evaluate the significance of the drug effect. When more than two groups were to be compared, the analysis of variance was performed (24).

Tissue preparation and ODC assay

At appropriate times after treatment, the animals were decapitated. The adrenal were removed, and the medulla was separated from the cortical tissue. The accuracy of the dissection was checked as previously described (1). Tissue from the two medullae or two cortices was pooled and homogenized in phosphate buffer 0.05 M, pH 6.8.

Determination of ODC activity by incubation of the 20,000 g supernatant with L-(^{14}C)ornithine and measurement of $^{14}CO_2$ liberated was performed by the method of Russell and Snyder (22) with some minor modifications (17). The reaction mixture contained 1 µCi of L-(^{14}C) ornithine 0.04 mM; pyridoxal 5'-phosphate, 0.05 mM; dithiothreitol, 1.0 mM; EDTA, 0.1 mM; and 100 µl of enzyme preparation in 0.5 ml total volume. The activity of the enzyme is always expressed as pmol $^{14}CO_2$ produced per mg of protein per 45 min of incubation at 37°.

RESULTS

Dose-response relationship for adrenal ODC after haloperidol

Administration of HLP to rats increased ODC activity of the adrenal

medulla during the initial 2.5 h in a dose-related manner (Figure 1). A significant rise (210% of control) occurred with 5 mg/kg HLP and the activity rose progressively with increasing doses, at least up to 20 mg/kg, where it was over 15 times control (Figure 1).

In the case of the adrenal cortex, ODC activity did not increase over control for any of the HLP doses studied. On the contrary, rats treated with 5 mg/kg showed a statistically significant decrease (49% of controls).

Dose-response for hypophysectomized rats

To determine if the pituitary gland is required for the HLP-induced increase in adrenomedullary ODC activity, the dose-effect relationship was studied in hypophysectomized rats. Hypophysectomy did not prevent the large increase produced by HLP in medullary ODC at 2.5 h. Rather, the hypophysectomized rats were more sensitive to HLP treatment, showing much larger percentage increases in enzymic activity. For example, with a dose of 5 mg/kg the increase over control went from 210% for intact to 565% for hypophysectomized rats (Figure 1).

As for the cortex, an apparent increase in ODC activity was observed after 10 mg/kg (Figure 1). However, contamination with small amounts of the highly responsive medullary tissue is undoubtedly responsible for this effect.

Time-course

The time-course of stimulation of medullary and cortical ODC activity by haloperidol was studied for two different doses, 5 mg/kg and 20 mg/kg

i.p. The highest enzyme activity in the medulla in this experiment was observed at 2.5 h for both doses studied; it then decreased with time. ODC activity of the cortex showed a different pattern in that it continued to increase with time, at least until 6.5 h (Figure 2). The slow response of adrenocortical ODC to HLP has been previously encountered with respect to aminophylline administration (6) and immobilization (18).

Effect of unilateral splanchnicotomy

Previous studies with cholinergic drugs and stressful stimuli (17, 18,21) have demonstrated that the early increase in adrenomedullary ODC activity produced by these agents is mediated by the sympathetic nervous system. To determine whether the HLP effect is similarly controlled, transection of the splanchnic nerve supplying the left adrenal gland was performed. Splanchnicotomy abolished completely the effect produced by HLP (20 mg/kg) in the adrenal medulla when comparisons of the left adrenal (denervated) versus right (intact) were made at 2.5 h, a time when the cortical tissue was unresponsive to the drug (Table I). Gross examination of the animals postmortem showed that there was no damage to the adrenal of the operated side, and no significant difference in the weight of the glands of the two sides.

DISCUSSION

The results of the present experiments clearly indicate that HLP markedly stimulates ODC activity in adrenal medulla of the rat, and that its largest effect as seen in these experiments occurs at 2.5 h after

the administration of the drug. The increase is greater when high doses are given, and it can be prevented by denervation of the adrenal medulla. In all previous reports, intact innervation to the adrenal was required in order for cold exposure (5,18), immobilization (18), reserpine (18) and cholinergic drugs (17,21) to produce increases in adrenomedullary ODC activity. In addition, the ganglionic blocker, mecamylamine, was found to lower the ODC increases induced by carbamylcholine (7). These increases in enzymic activity are preceded in most instances by a rise in the cyclic AMP levels and are paralleled in some cases by an increase in activity of cAMP-dependent protein kinase as shown by Byus and Russell (5). They used actinomycin D and cycloheximide to demonstrate that both RNA and protein synthesis are necessary for the increases in ODC activity observed by cold exposure and the administration of aminophylline. Moreover, kinetic studies of adrenomedullary ODC preparations from oxotremorine-treated and control animals have shown that there is no change in the K_m for the substrate L-ornithine, but an increase in V_{max} (unpublished results). These data suggest that HLP, a centrally acting drug, induces adrenomedullary ODC activity by a reflex increase in preganglionic nerve activity.

Indeed, a short-term treatment of animals with antipsychotic phenothiazines and HLP (3) or microinjection of HLP into the caudate nucleus (12) increases the firing rate of dopaminergic neurons in the substantia nigra, as detected with extracellular electrodes. Several studies have now shown that systemic administration of HLP also increases

biosynthesis and release of dopamine, that is, its turnover. Accumulation of O-methylated catecholamines in brain following neuroleptics, including HLP, was first demonstrated in mice by Carlsson and Linqvist (8). Accumulation of the acidic metabolite dihydroxyphenylacetic acid has also been detected in the substantia nigra and ventral tegmental area of rats (2,23).

The increase in firing rate of dopaminergic neurons caused by HLP is antagonized by apomorphine (4), presumably by its action at a presynaptic site. Our results previously demonstrated (1) a similar effect when piribedil was administered to rats pretreated with HLP and adrenomedullary ODC activity was measured at 2.5 h after the HLP was given.

The results with hypophysectomized rats also indicate that the pituitary gland can modulate the sensitivity of the animal to HLP, the operated rats showing a larger increase in adrenomedullary ODC than intact (non-operated) rats in response to the same amount of drug. Perry et al. (16) have recently reported that hypophysectomy increases the sensitivity of DA receptors, increasing the B_{max} of (^{3}H)spiroperidol-binding to striatal membranes as well as inducing hypersensitivity to apomorphine when stereotypy was assessed. Furthermore, it is clear that the presence of the pituitary gland is not required for the effect of HLP on medullary ODC activity.

In the case of the adrenal cortex, there is a delayed response of ODC to HLP: up to about 4.5 h for a dose of 20 mg/kg and 6.5 h for

5 mg/kg. These effects confirm our previous results (1) where HLP was tested for its blocking action against apomorphine and piribedil. In that work the increase of cortical activity was shown to be prevented by hypophysectomy. As HLP is known to release prolactin (10), adrenocorticotropic hormone and β-endorphin (11), it is conceivable that its delayed effect on the enzyme of the cortex is mediated by a specific peptide or peptides. Indeed, administration of ACTH (14,20), growth hormone (14), or prolactin (19,25) to hypophysectomized rats causes an increase in ODC activity of the adrenal in the rat. However, nothing is known about the mechanism of action of the hormones on adrenocortical ODC activity as all measurements have been done on homogenates of the whole adrenal gland. ACTH causes increases of cAMP in the adrenal, as well as ODC activity. The latter change seems to be independent of steroidogenesis, and it is blocked by cycloheximide and actinomycin D (20,15).

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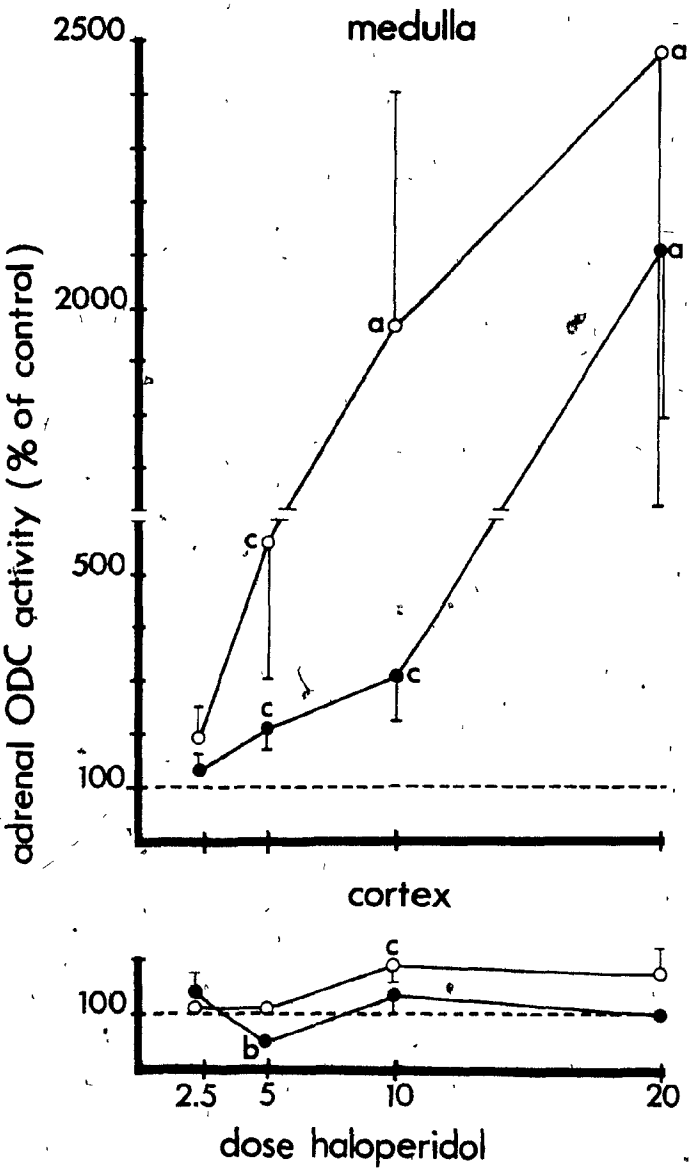

FIGURE 1

Dose-response of the effects of haloperidol (2.5, 5, 10 and 20 mg/kg i.p.) on ODC activity in the adrenal medulla (0 hypox, ● intact) and cortex (0 hypox, ● intact).. The animals, 4-5 per group, were killed 2.5 h after the drug. Values are expressed as mean ± SEM. Control values (100%) were 33 ± 3.7 and 25 ± 2.9 (for medulla) and 61 ± 4.2 and 16 ± 2.3 (cortex, intact and hypox, respectively) pmol $^{14}CO_2$/mg protein per 45 min. The significance of the difference from controls were a, $P < 0.001$; b, $P < 0.01$; and c, $P < 0.05$.

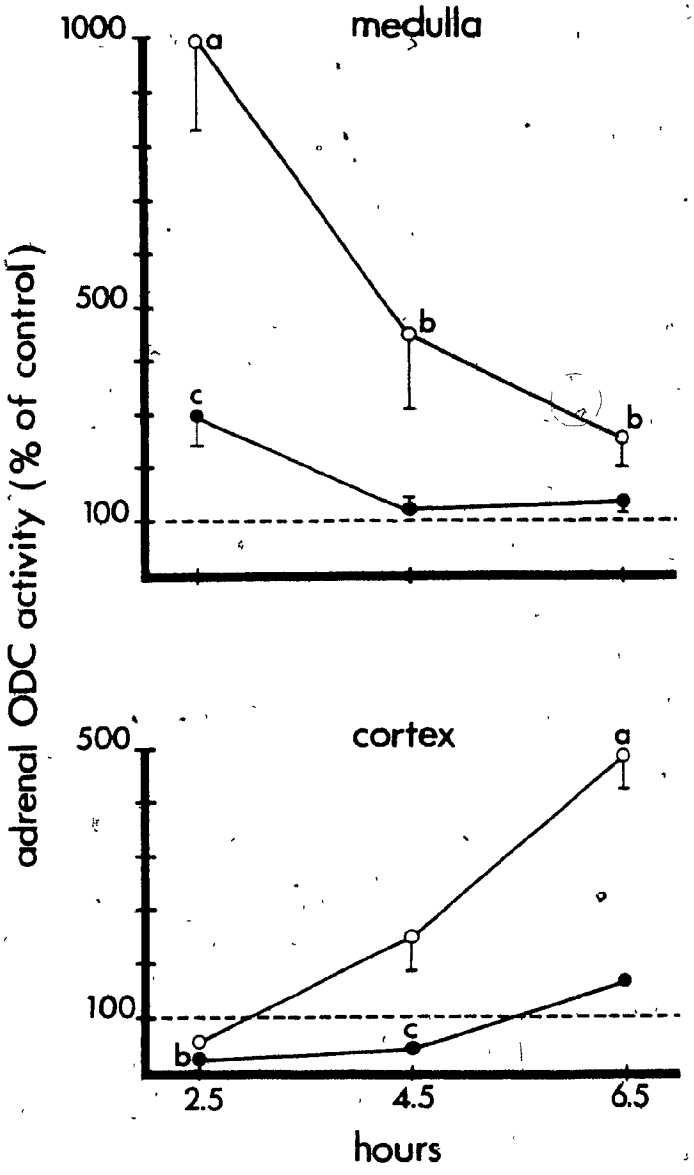

FIGURE 2

Time-response of the effect of haloperidol on ODC activity in the adrenal medulla (○ 20 mg/kg, ● 5 mg/kg i.p.) and cortex (○ 20 mg/kg, ● 5 mg/kg). The animals, 4-5 per group, were killed at different times after the drug. Control values (100%) ranged from 27 ± 3.4 (mean ± SEM) to 57 ± 4.2 for medulla, and from 40 ± 7.4 to 153 ± 46.6 for the cortex, in different experiments. These values are expressed in pmol $^{14}CO_2$/mg protein per 45 min. The significance of the difference from controls were a, $P < 0.001$; b, $P < 0.01$; and c, $P < 0.05$.

TABLE I. Effect of haloperidol on adrenal ODC activity of unilaterally splanchnicotomized rats

Treatment	Medulla		Cortex	
	Intact	Denervated	Intact	Denervated
Control	24 ± 1.5	24 ± 1.8	19 ± 7.6	42 ± 18.6
HLP	519 ± 188.4[a]	52 ± 13.0	14 ± 4.0	12 ± 2.9

Values shown are mean ± SEM for 4-5 determinations and are expressed as pmol $^{14}CO_2$ per mg protein per 45 min. Haloperidol (HLP) was administered i.p. in a dose of 20 mg/kg and rats were killed 2.5 h after.

[a] $P < 0.01$ for comparison with denervated medulla.

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CHAPTER IV

Adrenomedullary Ornithine Decarboxylase Activity: Its Use in Biochemical Mapping of the Origins of the Splanchnic Nerve in the Rat

SUMMARY

The activity of ornithine decarboxylase in the adrenal medulla of the rat can be induced transynaptically by the repeated administration of apomorphine. Unilateral section of one to four ventral spinal cord roots from T_4-T_{12} partially prevents this effect. Interruption of the intercostal nerves (T_7-T_{13}), with preservation of the autonomic innervation of the adrenal medulla, does not produce any alteration in the response of the medullary ODC to APM in the operated side as compared to the intact side. Dorsal root section at T_7-T_{10} leads to a small reduction, while section at T_2-T_4 has no effect at all. Thus, selective surgical interruption of spinal cord roots indicates that the bulk of splanchnic fibers mediating the transynaptic induction of adrenomedullary ornithine decarboxylase course in the ventral roots between T_7 and T_{10}. Dorsal rhizotomy demonstrates a modulatory role in this induction of afferent information to sympathoadrenal preganglionic neurons involved in innervation of the chromaffin cells.

INTRODUCTION

Investigation of the mechanisms regulating the activity of adrenal medullary enzymes, in particular tyrosine hydroxylase (EC 1.14.16.2) and ornithine decarboxylase (ODC; EC 4.1.1.17), has demonstrated the crucial role of nervous impulses to the chromaffin cells by way of the splanchnic nerve (12,17,22). More recent studies have turned to the detection of the prior influences in the central nervous system whose effects converge upon the preganglionic sympathetic neurons innervating the adrenal medulla, thereby regulating the activities of tyrosine hydroxylase (6,13, 14) and ODC (15,16). For example, in this laboratory it has been found that treatment of rats with dopaminergic agents increases the activity of these enzymes (1,13,14) and that this effect is mediated at a supraspinal level in the case of tyrosine hydroxylase (6). The present work is concerned with experiments in which ODC, the rate-limiting enzyme in polyamine biosynthesis (21) and one that seems to have an important role in early embryonal development (5), has been measured. This enzyme catalyzes the conversion of ornithine to putrescine (1,4-tetramethylenediamine). Its activity in the rat adrenal medulla can be stimulated not only by the administration of dopaminergic and cholinergic drugs (1,15,17) but also by subjecting the animals to stressors such as cold or immobilization (2,16). A complete transection of the splanchnic nerve supplying the adrenal gland virtually abolishes the increase in medullary ODC activity produced by all these agents (2,15,16,17).

The origin and course of the preganglionic fibers innervating the adrenal chromaffin cells of the rat have been examined in the past by different methods: Wallerian degeneration (11); chromaffin reaction (8); and the retrograde movements of the dye "True Blue" (10) and the enzyme horseradish peroxidase (18). The fibers passing to the adrenal medulla originate in the intermediolateral cell group of the spinal cord, the neurons constituting a long, continuous column from T_1 to L_1. The highest numbers of these cells are concentrated in the area between T_8 and T_{10} (18). We wished to learn if nerve impulses generated in the preganglionic neurons originating at different spinal levels contribute proportionally to the induction of the activity of medullary ODC. It was therefore decided to alter the sympathetic central output and input by cutting ventral and dorsal roots of the spinal cord, respectively, followed by attempts to stimulate adrenal medullary ODC activity by use of the dopaminergic agonist apomorphine (APM) (1,4,20).

MATERIALS AND METHODS

Male adult Sprague-Dawley rats (300 g), maintained with a light-dark cycle of 12:12 h, and given tap water and Purina Checkers ad libitum, were used throughout this work. Surgery was done under chloral hydrate anesthesia. In eight rats, the intercostal nerves from T_7 to T_{13} were cut on the left side at their immediate exit from the intercostal space; this preserved the autonomic innervation to the adrenal medulla (Figure 1D). In the remaining rats laminectomy was performed in order to cut ventral thoracic roots (T_4-T_{13}, in groups of 1 to 4 roots) (Figure 1A); dorsal roots (T_2-T_4, T_7-T_{10}) (Figure 1B); and combined

ventral and dorsal roots (T_7-T_{10}) (Figure 1C). The roots were identified according to Gelderd and Chopin (7). Five days postoperatively, the animals were injected with apomorphine.HCl (donated by Merck-Frosst Laboratories, Kirkland, P.Q.), 3 doses of 10 mg/kg s.c. at 1.5 h intervals (0, 1.5 and 3 h), or with the same volume of control vehicle. Rats were decapitated 4 h after the first injection of APM, and the adrenal glands were quickly removed and dissected at 4°. The portion of the tissue corresponding to one or two medullae was homogenized in 200 µl of phosphate buffer, 0.05 M, pH 6.8, with a motor-driven Teflon pestle. The 20,000 g supernatant was used for the ODC assay and determination of protein content (for details of tissue preparation and assay of ODC activity in vitro, see ref. 15). The activity of the enzyme is expressed as pmol $^{14}CO_2$ produced per mg of protein during 45 min incubation at 37°.

Standard statistical methods were used (19) in computing the data and establishing significant differences.

RESULTS

The ODC activity of adrenal medullary tissue was first determined in intact rats (200 g), receiving either saline or APM treatment. Tissue from the two glands was pooled. The results in Table I show that the low ODC activity of the tissue can be remarkably increased by the administration of APM, as previously demonstrated (1). Other control experiments are set out in that table. Animals with unilateral section of the ventral roots T_7-T_9 had ODC activity in the adrenal medullary tissue that hardly differed from that of the intact animals. Thus, the surgical operation alone had no effect on ODC activity under the described

conditions. Other rats underwent unilateral section of seven nerves in the intercostal space (Figure 1D, Table I) and were then given APM to stimulate ODC activity. There was a nine-fold increase over the controls, the values being similar for the adrenomedullary tissue of the operated and intact sides, and both similar to the activity in APM-treated intact animals (Table I).

Section of ventral roots of the spinal cord modified to varying extents the APM-induced increase of adrenomedullary ODC activity of the operated side as compared to the intact side. Section of the individual ventral roots T_8, T_9, and T_{10} led to attenuation of the response to APM by 21 to 29% (Table II; Figure 1A). Much greater reductions in activity were obtained by section of three or four roots. For example, with section of ventral roots T_7 to T_{10} there was a mean reduction of 61%. There was greater retention of the APM effect even with the more extensive lesions where the section was made further away from the T_7 to T_{10} region. With section of the ventral roots T_4 to T_5 there was essentially the same response as in control animals, i.e. equal induction of ODC activity on both sides. Thus, our procedure demonstrates that fibers mediating this particular functional activity of the splanchnic nerve have their origin in the spinal cord between segments T_4 and T_{12}, and interruption of ventral roots beyond these limits does not affect the adrenomedullary ODC response to the administration of APM.

The possible contribution by fibers, coursing in the spinal dorsal roots, to the regulation of ODC activity was also investigated. Section of four dorsal roots (T_7-T_{10}) led to 27% reduction in the medullary ODC

activity of the gland on the operated side following APM administration (Table II; Figure 1B). To assess the specificity of this effect, three dorsal roots in an area known to make little contribution to the innervation of the adrenal medulla, T_2-T_4, were cut in other rats. There was no difference in response of the medullary ODC between the two sides. The results with dorsal root section at T_7-T_{10} are, therefore, physiologically significant, and favor the concept of operation of segmental reflex arcs in the modulation of activity of sympathoadrenal preganglionic neurons (SAPN), or attest to the presence of efferent fibers coursing in dorsal roots (3).

Combined section of dorsal and ventral roots at T_7-T_{10} caused a mean decrease of 50% in medullary ODC activity of the adrenal on the operated side (Table II: Figure 1C). This is similar to what was observed when only the ventral roots in this region were severed (Figure 1A). The non-additive effect of dorsal and ventral root section again emphasizes the modulatory role of the afferent information reaching the spinal segments involved in innervation of the adrenal medulla.

DISCUSSION

Anatomical studies by Schramm et al. (18) and by Holets et al. (10) have shown that the sympathoadrenal preganglionic fibers are distributed from segment T_1 to segments L_1-L_2, with the largest concentration of cell bodies in the areas of T_7-T_{10} and T_7-T_8, respectively. We have shown that the increase of adrenal ODC activity provoked by APM reflects a distribution of fibers subserving this function between T_4 and T_{12}, with

the major segmental contributions at T_7 to T_{10} (Figure 1A).

The results with rats that have undergone section of dorsal roots T_7-T_{10} alone or along with the corresponding ventral roots make manifest the existence of a modulatory function of the afferent input in regard to the activities of SAPN (Figures 1B and 1C). This modulatory input enters the spinal cord by way of the autonomic pathways, for section of seven intercostal nerves (Table I; Figure 1D), leaving intact both afferent and efferent innervation of the adrenal medulla, does not alter in any way the response of medullary ODC to APM. The segmental integration of this modulatory phenomenon is shown by the results obtained in animals with section of dorsal roots at two different levels (Figure 1B). With section of dorsal roots at the higher level (T_2-T_4), where SAPN are essentially absent, no apparent reduction in the APM-induced effect on medullary ODC activity is observed. However, section of dorsal roots at T_7-T_{10} produces a considerable reduction in medullary ODC activity. As already mentioned, this is the area that concentrates most of the preganglionic fibers reaching the adrenal medulla. It seems likely that the activity of SAPN is sustained by descending afferent volleys, originating supraspinally (6), and modulated by the peripheral segmental afferent input that enters the spinal cord by way of autonomic pathways (Figure 1B). As reflected in our results, one may speculate that this input is mainly facilitatory.

Finally, we consider that the technique of root section used here in conjunction with a biochemical measurement of activity of adrenal

chromaffin tissue can be extended readily to other functions. The transynaptic induction of tyrosine hydroxylase, biosynthesis of catecholamines in the adrenal, and release of catecholamines under the influence of stress or insulin administration (23), come readily to mind. It would be interesting to determine whether the chromaffin cells receive innervation from the same segments of the spinal cord for all their many functions that respond to nervous influences, and how this innervation is differentiated with respect to the types of catecholamine-containing cells, a question to which Hirano and Niijina have addressed themselves (9). There is already a partial answer to the first question from the present work as well as that of others. Waki (23) has shown that the release of catecholamines from adrenal medulla in dog in response to electrical stimulation of ventral roots is mediated by fibers having their origin between T_5 and L_1 for epinephrine, and T_5 and T_{12} for norepinephrine, with the maximal effects around T_{10}. Stimulation of dorsal roots was ineffective in releasing catecholamines. Schramm has traced splanchnic fibers from the adrenal gland to the spinal cord by the retrograde movement of horseradish peroxidase; these fibers had a somewhat wider distribution (18) than SAPN mediating the transynaptic induction of adrenomedullary ODC in our work (Figure 1).

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TABLE I. ODC activity of adrenal medulla in various control groups of rats

Region of section	Treatment	No. of rats	ODC activity[a]
(Intact)	Control	4	41 ± 6.6
(Intact)	APM	5	318 ± 30.4*
Ventral roots, T_7-T_9	Control		
Intact side		4	40 ± 2.7
Operated side		4	46 ± 6.6
Intercostal nerves, T_7-T_{13}	APM		
Intact side		8	358 ± 38.3*
Operated side		8	356 ± 48.8*

Apomorphine (APM) was administered in 3 doses of 10 mg/kg i.p. or s.c. at 1.5 h intervals (0, 1.5 and 3 h) and rats were killed 4 h after the first injection.

[a] pmol $^{14}CO_2$/mg protein for 45 min of incubation; mean ± SEM is tabulated.

* $P < 0.001$ for comparison with control intact rats.

Legend to Table II

* Because the variance of the data tended to increase in proportion to the means of the groups, values for ODC activity were converted to their natural logarithms, a measure that provides a more nearly normal distribution. The effect of a particular unilateral treatment was then calculated as the difference between the ODC activities for the two sides. Analysis of variance (19) for 15 groups of rats, each group consisting of 4 to 10 animals, was based upon 14 degrees of freedom for "Groups" and 66 degrees of freedom for "Error determination". The corresponding variance was employed in calculating significant differences between groups. All logarithmic summary data were then transformed to the natural numbers, and these are set out in Table II as the weighted mean ratios of ODC activities on the two sides.

Significant differences for animals with ventral root dissections (*P < 0.05; **P < 0.001):

T_4-T_5 versus T_6-T_8*, T_7-T_{10}**, and T_{10}-T_{12}*;

T_6-T_8 versus T_7-T_{10}* and T_{13}*;

T_7-T_9 versus T_7-T_{10}*, T_9*, and T_{13}*;

T_7-T_{10} versus T_8*, T_9**, T_{10}**, T_{10}-T_{12}*, and T_{13}**;

T_{10}-T_{12} versus T_{13}*.

Significant differences at T_7-T_{10}:

V versus D* and D+V*;

D versus D+V*.

TABLE II. ODC activity in adrenomedullary tissue of rats with section of spinal roots in thoracic region, after treatment with apomorphine

Thoracic roots sectioned	V/D	No. of rats	Mean ratio* (as %) Operated side:Intact side
4-5	V	4	93
4-6	V	5	88
6-8	V	6	61
7-9	V	5	54
7-10	V	7	39
8	V	5	71
9	V	8	79
10	V	5	77
10-12	V	4	60
13	V	4	105
2-4	D	4	106
7-10	D	4	73
7-10	D+V	10	50

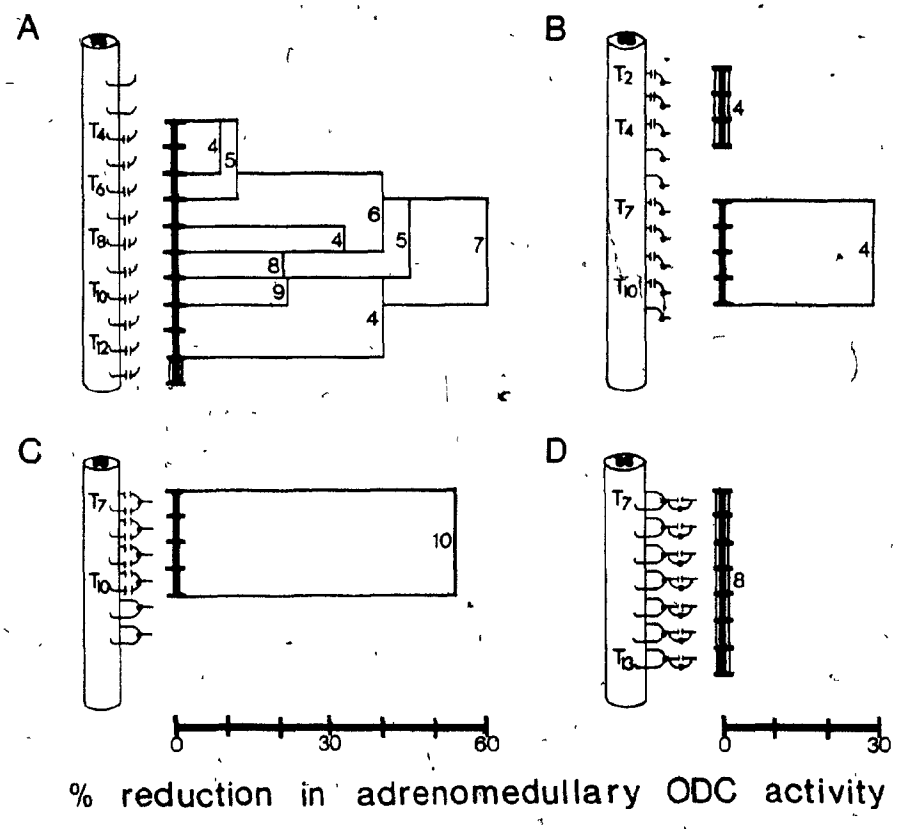

FIGURE 1

Percent reduction of APM-induced increase of ODC activity

Blocks represent mean values of the percent reduction in ODC activity of the left adrenal medulla in comparison to the right adrenal medulla (ipsilateral and contralateral to denervation, respectively) in response to APM. The drug was injected in 3 doses of 10 mg/kg s.c. at 0, 1.5 and 3 h. Rats were killed 4 h after the first dose. Numbers inside the blocks represent number of animals (4-10) in each group. Unilateral denervation was as follows: A, section of ventral spinal roots T4-T13 in groups of 1 to 4 roots; B, section of dorsal roots from T2-T4 and T7-T10; C, section of dorsal and ventral roots from T7-T10; and D, section of the intercostal nerves from T7-T13. In B, C and D the filled circles represent dorsal root ganglia. In D, additional dots associated with the intercostal nerves represent sympathetic chain ganglia.

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CHAPTER V

Central Dopaminergic Regulation of Adrenomedullary Ornithine Decarboxylase Activity

ABSTRACT

The administration of the two dopamine receptor agonists apomorphine (APM) and piribedil (PBD) to rats leads to an increase in ornithine decarboxylase (ODC) activity in the adrenal medulla. In this work, we have tried to elucidate the neural pathways involved in the regulation of this enzyme. The treatments involved are: unilateral splanchnicotomy, spinal cord section, intraventricular injection of the neurotoxin 6-hydroxydopamine, and section of the brain at various levels. Unilateral splanchnicotomy reduces very significantly the induction of ODC produced by either APM or PBD. Spinal cord section at either of two different levels (T_5 or T_2) also lowers the response to the drug. Intracerebroventricular injection of 6-hydroxydopamine, on the other hand, elevates the mean response to APM, although not to a statistically significant extent. Section of the mesencephalon well below the periaqueductal gray does not alter the response of adrenomedullary ODC to APM. Transection of the diencephalon almost prevents it whereas hypothalamic deafferentation and incomplete diencephalic transection potentiates the effect of this drug. These observations strongly suggest that adrenomedullary ODC activity is predominantly regulated by a central system, originating mainly in the diencephalon-telencephalon and including a facilitatory dopaminergic component.

INTRODUCTION

Recent studies in our laboratory have shown that the activity of ornithine decarboxylase (ODC, EC 4.1.1.17) in the adrenal medulla of the rat can be increased several fold by the administration of the dopamine agonists apomorphine (APM) and piribedil (PBD) (2). These effects are time-dependent for both drugs and dose-related for APM. That is, at least two doses of the drug (10 mg/kg) are necessary to increase ODC significantly over controls. The specificity of action of these drugs on dopamine receptors resulting in an increased adrenomedullary ODC activity was checked in the same work by the prior administration of haloperidol. A dose of 5 mg/kg of this dopamine-receptor blocker antagonized the effect of the drugs up to 4 h, but haloperidol itself in larger doses increased adrenomedullary ODC activity in a time-dependent manner (1). This latter action was prevented by denervation of the adrenal gland, as has been reported for ODC increases after cold exposure (11,42), immobilization and administration of reserpine (42,15) and cholinergic drugs (41,46).

In related work we have found that unilateral section of ventral spinal roots reduces the inductive effect of APM administration, the reduction depending upon the number of roots interrupted and their location in the thoracic region from T_4 to T_{12} (3). All these results suggest a crucial role of central innervation in determining the induction of ODC activity.

We have now sought to define further the central dopaminergic system involved in the APM-induced increase of adrenomedullary ODC activity. In this regard we have kept in mind the earlier result, namely that another medullary enzyme, tyrosine hydroxylase (tyrosine 3-monooxygenase, EC 1.14.16.2), is also affected by a central dopaminergic system (39) lying rostral to the thoracic cord (19).

EXPERIMENTAL PROCEDURES

Materials

Pyridoxal 5'-phosphate (crystalline), DL-dithiothreitol and 6-hydroxydopamine.HBr (6-OHDA) were purchased from Sigma Chemical Co. (St. Louis, MO). Apomorphine.HCl was obtained from F.E. Cornell and Co. (Montreal, P.Q.), and chloral hydrate from Fisher Scientific Co. (Montreal, P.Q.). Synthetic ACTH (Synacthen depot) was purchased from Ciba-Geigy (Dorval, P.Q.). Piribedil (PBD) was a gift of Laboratoires Servier (Neuilly-sur-Seine, France). L-(1-^{14}C)Ornithine and 2,5-diphenyloxazole were purchased from New England Nuclear (Boston, MA). Desmethylimipramine (desipramine) was donated by Ciba-Geigy (Dorval, P.Q.). All other chemicals (analytical quality) were obtained from standard commercial sources.

Animals

Male Sprague-Dawley rats weighing 203 g ± 1.9 (SEM), used in various experiments, except where the weight is otherwise specified, were obtained from Canadian Breeding Farms and Laboratories Ltd., St. Constant,

P.Q. They were kept in the animal room in individual wire cages under a light-dark cycle of 12:12 h with tap water and Purina Checkers ad libitum.

Apomorphine.HCl was dissolved, 3.3 mg/ml, in 0.1% solution of sodium metabisulfite to prevent oxidation. Piribedil was suspended, 16.7 mg/ml, in a 1% solution of methyl cellulose. Desipramine, 6.7 mg/ml, was dissolved in 0.9% saline. The three drugs were titrated with 0.1 N NaOH to around pH 6 and injected in a volume of 3 ml/kg body weight. 6-OHDA was dissolved, 12.5 mg/ml, in a solution of ascorbic acid, 1 mg/ml, in 0.9% saline, and was injected into the right lateral ventricle in a volume of 10 µl (containing 125 µg) over a 2 min period. The dosage of the drugs and timing of the injections are indicated in the legends to the figures and tables.

Surgical procedures

All surgery was performed in this laboratory under chloral hydrate anesthesia (300 mg/kg i.p.). For injection of 6-OHDA into the right lateral ventricle and sections of the brain, the rats were placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). Sections of the diencephalon and mesencephalon were carried out in 267 g ± 2.3 rats with a sharp stainless steel knife, 1 mm wide and 15 mm long, mounted on the stereotaxic carrier. The coordinates of König and Klippel (30) were used to locate intracerebral targets.

Unilateral (left) splanchnicotomy in rats weighing 200 g ± 2.7 was performed with the aid of a dissection microscope after a midline laparotomy. The vascular supply to the gland was left intact, as could be observed under the microscope during the operation.

Spinal cord transection was carried out in rats weighing 312 g ± 1.5, with fine scissors after posterior laminectomy of the vertebrae corresponding to T_2 or T_5 spinal levels. Some rats underwent laminectomy and section of the dura, but not section of the cord; these are termed "sham-operated rats".

Intraventricular injection of 6-OHDA was carried out in rats weighing 243 g ± 2.4, pretreated 1 h earlier with desipramine, 20 mg/kg. Animals were placed in a stereotaxic apparatus, and they were injected with a Hamilton microsyringe into the right lateral ventricle. Sham-operated controls were injected with the same volume (10 µl) of carrier solution. All brains were examined postmortem to check the trajectory of the needle into the ventricle.

Incomplete mesencephalic transection. A fissure was drilled 6 mm long transverse to the midline of the skull and the knife assembly was lowered into the brain in the sagittal plane, with the following coordinates for the tip of the knife: A 0.5, L ±2.5, V -2 (cf. Figure 1A).

Complete diencephalic transection. A fissure 9 mm long was drilled as above. The knife was lowered with the following coordinates: A 3, L ±4, V -3.0. The lesion is illustrated in Figure 1B.

Hypothalamic island. This procedure was performed with modifications of the technique described by Halász and Pupp (24). The knife assembly used in these operations consisted of a stainless steel wire, inside a 23-gauge cannula, and sharpened at its exposed end. The inverted L-shaped knife formed a 90° angle and its sides measured 2 mm by 3.8 mm. Coordinates used were: A 6.5, L 0, V -3. After the knife point had reached its target, the knife was turned 90° to the right and left by means of the handle, thus providing a semicircular cut at the level of the optic chiasma, with its concavity directed posteriorly. With the knife turned laterally 2 mm to the right, the assembly was lowered ventrally so that the tip would be at these coordinates: A 2.5, L 2, and V -3.4 to -3.6. At this new position the knife was turned through 180° clockwise so that another semicircular lesion was made. The complete circle then included an area extending from the optic chiasma anteriorly to the midmammillary bodies posteriorly. The knife assembly was later removed from the brain with the blade in the sagittal plane. The lesion is illustrated in Figure 1D.

Incomplete diencephalic transection. A fissure 4 mm in length was drilled transverse to the midline at the plane corresponding to the entrance of the knife. The carrier was set at 20° of inclination in relation to the anteroposterior plane, and the tip of the knife was directed to the target with the following coordinates: A 4.3, L 1.5, V -3.8 to -4.2 in different animals (to touch the base of the skull).

The knife was then lowered at 1.5 mm right, and moved with slow motion to 1.5 mm left of the midline.

There were two groups of sham-operated controls. In the first of these, the knife used for transection of the diencephalon was lowered into the brain 2 mm from the scalp and a transverse section was made in the same way as in lesioned animals. In the second group of sham-operated rats the knife used for isolating the hypothalamic island was lowered intracerebrally, but no section was made. The coordinates for the tip of the knife were: A 6.5, L 0, V -3.

Animals with brain sections were used 24 h after the operation, and those with splanchnicotomy, spinal cord transection and intraventricular injection between 4 and 8 days after surgery. On the day of the experiment the rats were injected with either APM (3 doses of 10 mg/kg i.p.) given at 0, 1.5 and 3 h or PBD (one dose of 50 mg/kg i.p.). The rats were killed by decapitation 4 h after the initial injection.

Preparation of the tissue

After decapitation of the rats, the adrenal glands were quickly removed, freed of capsular tissue and weighed on a torsion balance.

The dissection of the glands to separate medullary tissue from cortex was carried out at 4° with the aid of a magnifying lamp. The extent of contamination of medullary tissue with cortex was estimated to be about 15%; this was based upon measurement of corticosteroids in both adrenal portions by a microfluorometric method (21) as previously described (2).

The portion of the tissue corresponding to two medullae was pooled and homogenized in 200 µl of sodium-potassium phosphate buffer, 0.05 M, pH 6.8, with a motor-driven Teflon homogenizer. The homogenate was then centrifuged at 20,000 g for 20 min, and the supernatant fraction was taken for use in the assay of ODC activity.

Ornithine decarboxylase assay

ODC was determined in an assay system that combined elements of the assays described by Russell and Snyder (47), and Jänne and Williams-Ashman (27), with some modifications. In a final volume of 0.5 ml, 100 µl of the 20,000 g supernatant was incubated with the indicated final concentrations of reagents: phosphate buffer, pH 6.8, 50 mM; pyridoxal 5'-phosphate, 0.05 mM; dithiothreitol, 1.0 mM; EDTA, 0.1 mM; and 1 µCi of L(1-^{14}C)ornithine, 0.04 mM. The incubation mixture was contained in a 25 ml conical flask fitted with a plastic well and sealed by a rubber stopper. After 10 min preincubation at 37°, the reaction was initiated by adding the radioactive ornithine. After incubation for 45 min more, the reaction was terminated by the addition of 0.5 ml of 5N sulfuric acid, with an additional 60 min allowed for the release of all the $^{14}CO_2$ formed. The $^{14}CO_2$ was trapped in a filter paper (2 cm^2, Whatman no. 3) impregnated with 100 µl of a mixture of ethyleneglycol monomethyl ether and monoethanolamine (2:1, v/v). At the end of the incubation, the filter papers were transferred to counting vials containing 10 ml of a mixture of toluene and ethyleneglycol monomethyl ether (2:1, v/v)

containing 0.4% 2,5-diphenyloxazole. Radioactivity was measured in a Beckman liquid scintillation spectrometer.

Protein content of the adrenomedullary supernatant was measured by the method of Lowry et al. (33). ODC activity is expressed as pmol of $^{14}CO_2$ produced per milligram of protein during 45 min incubation.

Histological examination of the brain

The rat brains were fixed in 10% formaldehyde in saline solution and serial sections were cut in some of them to check the extent of the lesion. The rest of the brains were simply sectioned in a sagittal plane (midline) and photographs were taken directly.

Statistical procedures

Data in the tables and figures are represented as mean ± standard error of the mean (SEM). As the variance tended to vary directly with the size of the mean, individual values were transformed logarithmically prior to calculation of statistics used in assessing the significance of the data, i.e. Student's t-test in comparing two means and Fisher's F ratio in the analysis of variance (51).

RESULTS

The effect of dopamine agonists on adrenomedullary ODC activity

The repeated administration of APM to rats (10 mg/kg i.p. at 0, 1.5 and 3 h) results in a large increase in adrenomedullary ODC activity over control values 4 h after the first injection (Table I) as previously described by Almazan et al. (2). A single large dose of PBD (50 mg/kg

i.p.) produces a similar effect. Animals exhibited stereotyped behavior with apomorphine (52).

To determine how important innervation of the adrenal medulla is for the increase in ODC activity the left adrenal gland was denervated. The activity of the right adrenal medulla (innervation intact) served as control. Six to eight days after splanchnicotomy the animals were injected as in the previous unoperated groups. At the time of killing the rats, the weights of the denervated and innervated adrenals were almost identical (respective mean ± SEM were 14 ± 0.3 mg and 13.8 ± 0.2 mg, number of pairs = 34), as is the case in intact animals.

The results show that the basal adrenomedullary ODC activities in rats without drug treatment were almost identical for the denervated and innervated adrenals (Figure 2). However, differences appeared in animals receiving dopaminergic agonists: on the denervated side of APM-treated rats the activity was 2.5-fold greater than in the controls, and PBD provoked even larger increases. But these responses were far smaller than in the innervated gland of rats given either of the two agonists (Figure 2).

A comparison of the results in Table I and Figure 2 shows that the innervated gland of unilaterally splanchnicotomized rats is more sensitive to APM and PBD than is the adrenal of intact rats, with respect to medullary ODC activity. This suggests the operation of some compensatory mechanism when one of the splanchnic nerves is severed. Alternatively, the response mechanism may be sensitized by surgical trauma, even 4-8 days

after operation. A similar effect has been observed in rats given oxotremorine (41).

Inspection of the SEM bars in Figure 2 shows how large the variation in response to PBD is in these animals. This has been previously observed with this drug, in respect to responses of both adrenal medullary and cortical ODC activity (2). The variability may arise from the fact that PBD is administered as a suspension, and its absorption and circulation through the organism is erratic. Because its dopamine-like activity depends upon its metabolism to the catecholic derivative, 1-(3,4-dihydroxybenzyl)-4-(2-pyrimidyl)piperazine (14), variations in the rate of this conversion from animal to animal may also contribute to the large SEM.

Effect of ACTH on adrenomedullary and adrenocortical ODC activity: time-course

The effects of ACTH administration have been studied in hypophysectomized rats in order to determine if the release of this hormone from the anterior pituitary has any role in the small increase produced by APM and PBD in the denervated adrenal medulla. Five days after hypophysectomy, experimental animals received one subcutaneous injection of synthetic ACTH (10 IU or 0.1 mg/rat); controls received 0.1 ml of a 1% solution of methyl cellulose. To study the time-course, animals were killed every two hours up to 12 h, along with two additional groups at 18 h and 24 h after ACTH. As can be observed in Figure 3, adrenomedullary

ODC did not respond to ACTH administration in the time-course studied. In contrast, the cortical enzyme showed a significant increase at 6 h, rising to a peak at 12 h. Twenty-four hours after the administration of ACTH, the cortical enzyme still showed higher values than controls.

To see if the basal levels of ODC activity were affected by hypophysectomy, a group of sham-operated controls were included in the study. Table II shows that basal medullary ODC activity is not altered by hypophysectomy. Cortical ODC, however, is significantly lowered as compared to sham-operated controls. The same table displays the effect of two different doses of ACTH: 10 and 20 IU. A slightly higher mean value was obtained with 20 IU but this was not significantly different from that obtained with 10 IU. These results agree with the report of Levine et al. (31,32), who estimated a maximal dose of 10 IU ACTH for induction of adrenal ODC.

Effect of spinal cord transection on adrenomedullary ODC activity

The data obtained with unilaterally splanchnicotomized rats suggests that the mechanism of action of APM is largely central. In an effort to trace back the neural pathway from the adrenal gland to that central site of action of the drug the spinal cord was sectioned at T_2 and T_5, segmental levels that are rostral to the main roots of origin of the splanchnic fibers innervating the adrenal medulla (3,49). The results in Table 3 show that transection of the cord at T_5 of itself led to an increase ($P < 0.01$) in adrenomedullary ODC activity as compared to

sham-operated controls, an effect that was not observed when the section was carried out at T_2. The administration of APM produced significant increases in ODC activity of the adrenal medulla in all groups studied: cord-transected at both levels and sham-operated rats. The level of the operation seemed to make a quantitative difference in the response. Thus, rats with a sham-operation at T_2 showed a much larger increase over controls than those sham-operated at T_5. Animals sham-operated at T_2 may be more sensitive to the drug than at T_5, because the surgery is more stressful: the animals bleed more because the vertebra is very deep (T_2 represents the lowest point of the cervicothoracic curvature in the rat); furthermore, the muscles incised are more important at T_2 for movement of upper limbs and neck. By contrast, after section of the cord at T_2 there was a much smaller response of adrenomedullary ODC activity to APM than after section at T_5 (117 vs. 249). These results suggest that the APM-induced increase in adrenomedullary ODC activity originates mostly from an area rostral to the second thoracic segment of the spinal cord. However, a propiospinal mechanism seems to be operating as well, since section of the cord did not completely prevent the rise in medullary ODC produced by APM.

Effect of intraventricular 6-hydroxydopamine

Injection of the neurotoxin 6-OHDA into the right lateral ventricle, one hour after administering desipramine intraperitoneally to rats, selectively destroys dopaminergic neurons (8). This treatment did not

have a significant effect on adrenomedullary ODC activity (Table IV, Controls). Administration of apomorphine to rats 6-8 days after 6-OHDA led to large increases in ODC activity. The mean response was higher than in the control group, but because of the large variation in the individual values the difference could not be judged statistically significant.

The rats receiving 6-OHDA showed more intense stereotyped behavior after the injection of APM.

Effect of section of the brain at different levels

Sham-operated controls. Twenty-four hours after surgery, brain-operated controls showed ODC basal levels (Table V, Line 1) comparable to those of intact non-operated controls (Table I). The administration of APM produced a 5.5-fold increase over controls, similar to that produced in intact rats.

Incomplete mesencephalic transection. Transection of the midbrain well below the periaqueductal gray matter produced no change in the basal levels of adrenomedullary ODC activity. The data were pooled with those obtained in sham-operated controls since they were essentially the same. The administration of APM to this group of animals produced an elevation in the activity of ODC similar to that in sham-operated rats (Table V).

Complete diencephalic transection. In order to determine the site of action of APM for the induction of adrenomedullary ODC, complete

transection of the diencephalon was carried out. This lesion isolates the forebrain dopaminergic nerve terminals from their origin in the mesencephalic centers (substantia nigra and ventral tegmental area) (36). The results with rats operated at this level are shown in Table V. Two important consequences of this operation were observed. First of all, the basal activity of adrenomedullary ODC was significantly lower in such animals than in sham controls. Secondly, the large increases produced by APM in intact (Table I) and sham-operated (Table V) rats were almost, but not completely, abolished. The tripling of ODC activity produced by APM, though small by comparison with the induction of enzyme in sham-operated rats, was nevertheless significant ($P < 0.01$).

The data suggest then that APM acts in at least two central sites in the induction of adrenomedullary ODC activity. One site would lie below the diencephalic level, and might involve stimulation of descending nigrospinal or spinal neurons directly. But the full effect of APM, as seen in intact animals, would require the connection of mesencephalon-diencephalon with forebrain structures.

Hypothalamic island and incomplete diencephalic transection. Because the hypothalamus exerts an important regulatory influence on the autonomic nervous system, it was of interest to isolate surgically this region from the rest of the brain. A preparation of this kind could indicate whether the descending pathways mediating the APM-induced increase in adrenomedullary ODC pass through the hypothalamus. This

hypothalamic island, extending anteroposteriorly from the nucleus suprachiasmaticus to the posterior mammillary nucleus, produced no change in resting adrenomedullary ODC activity as compared to sham-operated controls. The administration of APM to these rats produced a 10-fold increase in ODC. These values were significantly higher than those obtained in sham-operated rats treated with the drug. The second type of lesion (complete dorsoventral section, but extending only 1.5 mm to each side of the midline), which extends from the rostral part of the superior colliculus ventrally to the medial hypothalamus, produced similar results: no alteration in resting ODC activity and a potentiation of the APM-induced increase.

DISCUSSION

Several observations suggest that central dopaminergic mechanisms are involved in the regulation of adrenomedullary ODC activity: first, the increase in adrenomedullary ODC produced by the agonists APM and PBD can be antagonized by haloperidol (2); second, transection of ventral spinal roots from T_4 to T_{12} reduces the effect of APM (3); and third, the increase caused by a large dose of haloperidol can be prevented by denervation of the adrenal (1). In this work, we have tried to define these pathways by the following surgical treatments: unilateral splanchnicotomy, transection of the cord at T_2 or T_5, and transection of the brain at two different levels (mesencephalic, diencephalic). We have also employed intraventricular administration of the neurotoxin 6-OHDA.

The increase in adrenomedullary ODC activity provoked by the administration of either APM or PBD was largely prevented by denervation of the adrenal (Figure 2). There was, however, a significant induction of enzyme on the denervated side of both APM and PBD-treated rats as compared to controls. In contrast, the effects of oxotremorine (41), carbamylcholine (46), reserpine (42) and cold exposure (11,42) are completely abolished by splanchnicotomy. This small, but significant, increase of ODC activity of the denervated gland could result if: (a) the adrenal glands were not completely denervated; (b) some humoral component is responsible for the increase; or (c) the drugs have a direct action on the adrenal medulla. Local effects of APM and PBD on adrenal medulla are unlikely because of the failure of the drugs to cause any increase of ODC in vitro (2). Results obtained earlier in our laboratory lend support to the second suggestion, inasmuch as the response of adrenomedullary ODC activity to APM was considerably reduced in hypophysectomized rats (2). Also, APM and other dopamine agonists cause an increase in the levels of circulating corticosterone in the rat (18,29). Additionally, the activity of another inducible enzyme of the adrenal medulla, phenylethanolamine-N-methyl-transferase, can be markedly reduced by hypophysectomy (55) and then restored to normal values by administration of ACTH or glucocorticoids. Hence, it was of interest to see whether ACTH has an effect on adrenomedullary ODC of hypophysectomized rats. Administration of 10 IU of synthetic ACTH

produced no increase in adrenal medullary ODC in the time-course studied (1-24 h). Basal levels of adrenomedullary ODC were similar to those observed in sham-operated controls. In contrast, cortical values of controls were significantly lower than sham, and ACTH administration caused a large time-dependent increase. The latter results agree with earlier reports of the effects of ACTH on ODC activity of the whole adrenal (31,32,44). These results exclude any possible obligatory role for ACTH in the APM-induced increase in adrenomedullary ODC activity. Growth hormone and prolactin are also said to cause a very small increase in whole adrenal ODC of hypophysectomized rats (31,43,53), but their mediation of the APM effect in the medulla is unlikely because growth hormone is not released by high doses of APM (37) and this drug inhibits release of prolactin both in vivo and in vitro (10,50). Finally, the possible participation of other peptides cannot be altogether excluded as β-endorphin is released from the anterior pituitary gland together with ACTH by different stimuli (4,22).

Section of the spinal cord at two different levels (T_5 or T_2) served to isolate sympathoadrenal preganglionic neurons from their supraspinal connections. This operation led to a large reduction in the inducibility of medullary ODC by treatment with APM. Cord section at T_2 produced a greater reduction in the efficacy of APM with respect to induction of ODC than section at T_5. These results suggest that the magnitude of transynaptic induction is proportional to the number of

sympathoadrenal preganglionic neurons that remain intact above the lesion, as previously demonstrated with section of ventral spinal roots (3). One can conclude that the APM-induced increase of adrenomedullary ODC activity originates mostly from an area rostral to the second thoracic segment of the spinal cord. As with splanchnicotomy, the effect of APM on medullary ODC is not completely abolished by cord section. Indeed, highly significant increases are still observed: 3-fold higher than spinal control with section at T_5, and 5-fold with section at T_2. These effects may be due to the action of the drug on dopamine receptors in the spinal cord. There is ample evidence for dopaminergic neurons in the spinal cord of the cat (17,6) and the rat (12,34), as well as for specific dopamine receptors there, binding ^{3}H-haloperidol in cat (13) and rat (16). Additionally, the steady state content of dopamine and its turnover rate in the spinal cord of the rat decreases in a rostro-caudal manner (28). Interestingly, a dopamine-activated adenylate cyclase has been identified in membrane fractions of the rat spinal cord (20) which is activated by APM and blocked by haloperidol. Following the transection of the cord, this adenylate cyclase becomes more sensitive to dopamine below the transection. Because we obtained a higher relative increase in ODC when the transection is carried out at T_2 than at T_5 (Table III), we may also suggest that dopamine receptors below the transection become more sensitive to APM.

Similarly, denervation supersensitivity to serotonin has been reported to develop after spinal cord transection (5).

To characterize further the dopaminergic pathways involved in the regulation of adrenomedullary ODC activity, the neurotoxin 6-OHDA was administered intraventricularly to rats pretreated with desipramine. Destruction of dopamine-containing nerve terminals (8,25) did not prevent the dopamine agonist APM from inducing an increase in adrenomedullary ODC activity. Similar results were obtained by Quik and Sourkes (39) with adrenal tyrosine hydroxylase activity. The explanation might be that, while the neurotoxin damages mainly presynaptic nerve endings (54) APM is acting postsynaptically on receptors unaffected by the neurotoxin or rendered supersensitive by destruction of the presynaptic fibers normally communicating with them. In fact, we observed enhanced stereotyped behavior with APM, as well as a mean enhancement of the APM effect on adrenomedullary ODC activity of animals treated with 6-OHDA.

A preliminary attempt has been made to localize cerebral centers responsible for the control of the APM-induced increase in adrenomedullary ODC activity by gross separation of large brain areas. Transection of the brain at the level of the mesencephalon below the periaqueductal gray matter, did not alter the response of adrenomedullary ODC to APM. These data lead us to conclude that the pathway mediating the effect is not located in the dorsal part of the midbrain. Complete transection of the posterior diencephalon, which isolates most of the diencephalon

and forebrain from posterior structures, produced an effect similar to section of the cord and splanchnicotomy, i.e. almost complete blockade of the APM effect on adrenomedullary ODC activity. This result then suggests that the APM-induced increase in adrenomedullary ODC activity is mediated at the level of the rostral diencephalon-telencephalon. As already proposed, APM seems to be acting to a small extent also below the level of the section. Descending dopaminergic pathways from the substantia nigra to the reticular formation (23) and from the diencephalic A_{11} dopamine cell group to the spinal cord in the rat (26,7) have been described by electrophysiological and histofluorescence techniques, respectively.

Interestingly, section of the diencephalon at the level of the medial hypothalamus and extending laterally 1.5 mm from the midline, along with complete deafferentation of the hypothalamus to produce a hypothalamo-pituitary island, permitted not only maintenance of the effect of APM on adrenomedullary ODC activity, but even its potentiation. The data suggest that pathways involved in this inductive phenomenon must be travelling more laterally than 1.5 - 2 mm from the midline at the level of the hypothalamus and, furthermore, that these lesions may sever some inhibitory pathway originating at the level of the hypothalamus or in passage there. The existence of a direct hypothalamic-autonomic nervous pathway has been demonstrated (48).

Because of the demonstrated role of a serotonergic pathway originating in the medial raphe nucleus in the regulation of adrenal tyrosine hydroxylase activity (9,39,40), our attention turned to the possibility that the potentiation observed after the lesions described in this paper might also be due to destruction of an inhibitory serotonergic system. Anatomical (35,38) and electrophysiological (35) studies have provided strong evidence for a serotonergic pathway emanating from the dorsal raphe nucleus and terminating in the caudate-putamen (45). Destruction of that serotonergic nucleus by electrolytic lesions increases the dopamine content in the striatum. Further experiments would be needed to determine if serotonin does play a role or not. Because both lesions, hypothalamic deafferentation and incomplete diencephalic transection, must sever some dopaminergic neurons, another possibility to explain the potentiation effect must be considered. This is a denervation supersensitivity, previously proposed to explain the effects caused by 6-OHDA on the APM-induced increase in ODC activity.

In conclusion, it has been demonstrated that adrenomedullary ODC activity can be increased by the administration of the dopamine receptor agonists APM and PBD. This increase is dose-related and time-dependent (2). Unilateral splanchnicotomy, section of the spinal cord and transection of the diencephalon result in a very large decrease in the response of adrenomedullary ODC to APM. These observations strongly suggest that adrenomedullary ODC activity is predominantly regulated by

a central system originating mainly in the diencephalon-telencephalon, and containing a facilitatory dopaminergic component. There is some evidence that a propiospinal dopaminergic pathway also participates in the regulation.

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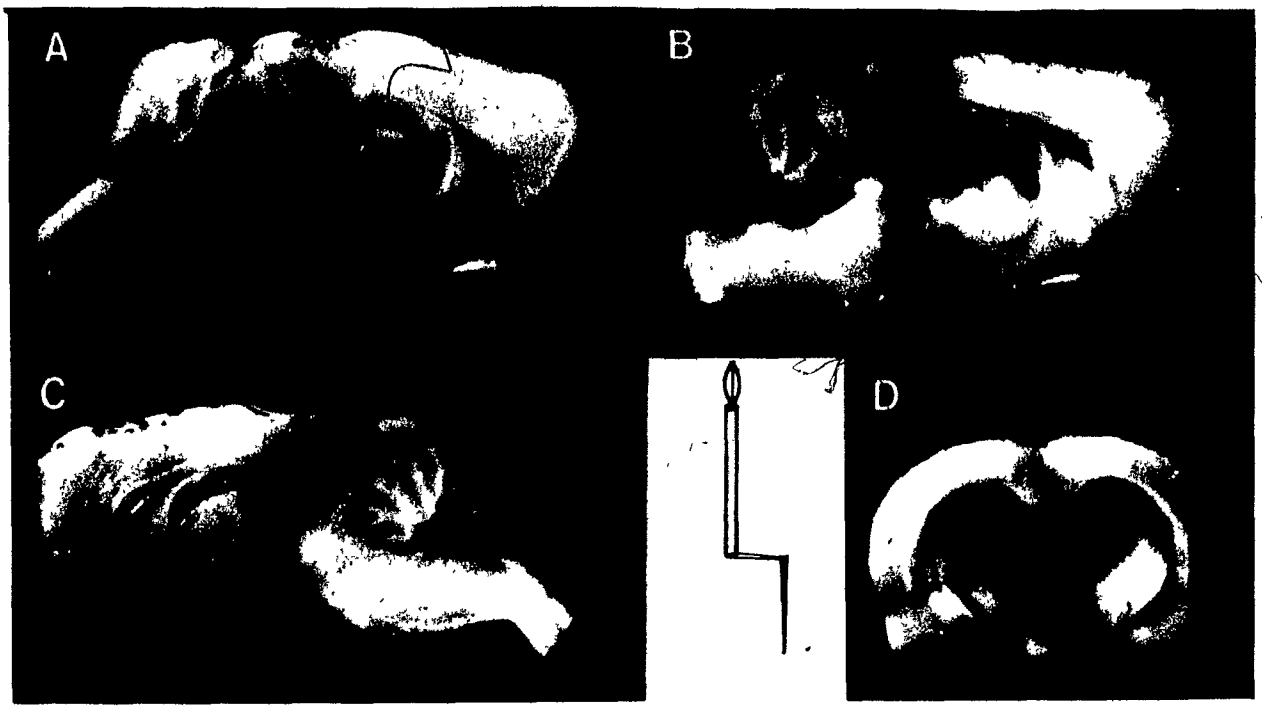

FIGURE 1

Representative lesions caused by: A, mesencephalic section (sagittal view); B, diencephalic transection (sagittal view); C, incomplete diencephalic (sagittal view) transection; and D, hypothalamic island (coronal view). Knife assembly shown in diagram.

TABLE I Effect of apomorphine and piribedil on adrenomedullary ODC activity in intact rats.

Treatment	Control		Drug-treated		P
APM	40.9 ± 3.7	(7)	311.1 ± 19.7	(8)	< 0.001
PBD	42.4 ± 3.1	(7)	411.0 ± 70.3	(7)	< 0.001

Apomorphine (APM) was administered i.p. in a dose of 10 mg/kg at 0, 1.5 and 3 h. Animals were killed 4 h after the first injection. Piribedil (PBD) was injected i.p. in one dose of 50 mg/kg. Animals were killed 4 h after. Figures are given as pmol CO_2 per mg protein per 45 min and represent the mean ± SEM for the number of observations in parentheses. The P value indicates the level of significance for the difference between the control and treated groups (Student's t-test).

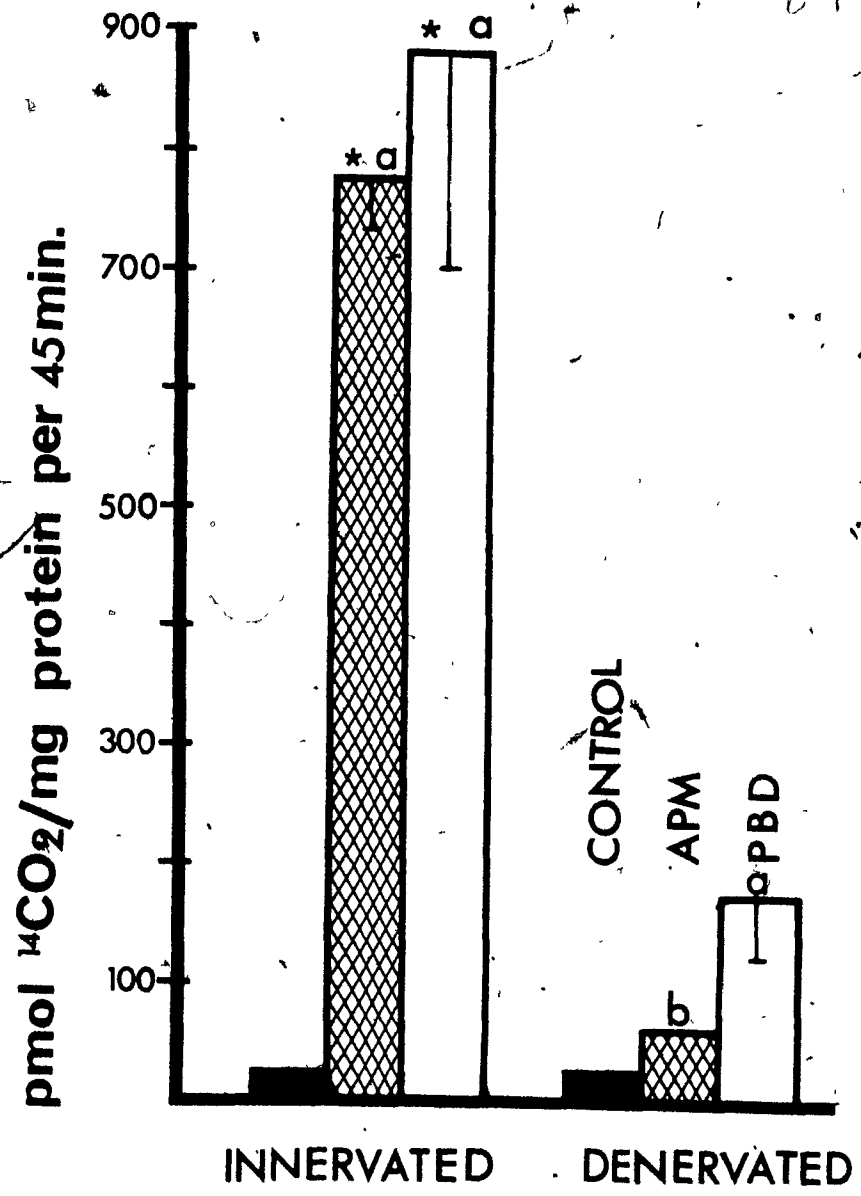

FIGURE 2

Effect of apomorphine (APM) and piribedil (PBD) on adrenomedullary ODC activity in unilaterally splanchnicotomized rats. The animals were decapitated 4 h after the first of 3 injections of APM (10 mg/kg each) or 1 dose of PBD (50 mg/kg i.p.). The bars represent the mean ODC activity ± SEM for 5-7 animals in each group. Control values were 24 ± 1.5 and 24 ± 1.4 pmol CO_2 per mg protein per 45 min for denervated and innervated adrenal medulla, respectively.
*$P < 0.001$ for comparison of indicated mean with contralateral side
$^aP < 0.001$ for comparison of indicated mean with ipsilateral side
$^bP < 0.01$ for comparison of indicated mean with ipsilateral side.

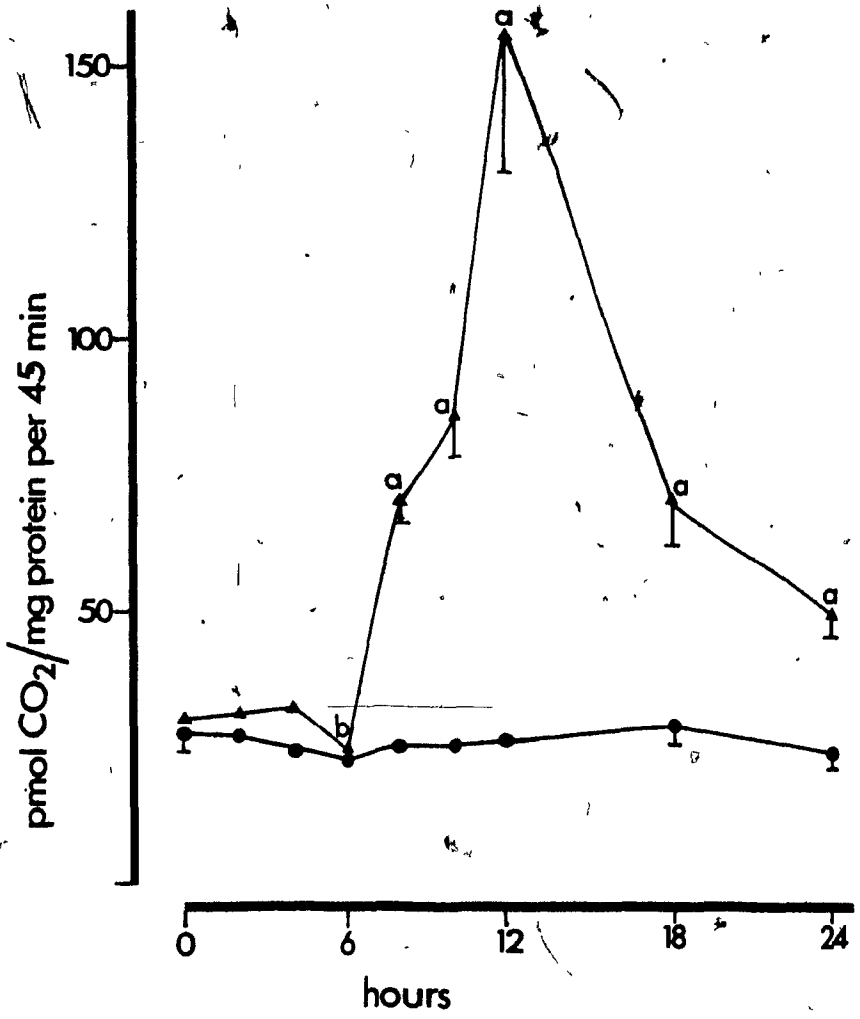

FIGURE 3

Time-course of stimulation of adrenomedullary (●) and adrenocortical (▲) ODC following subcutaneous administration of 10 IU of ACTH. Each point represents the mean response of 4-6 animals.

$^{a}P < 0.001$, and $^{b}P < 0.05$ for comparison of indicated mean with control.

TABLE II Effect of hypophysectomy with and without ACTH on adrenomedullary and cortical ODC activity

Treatment	ACTH	Medulla	Cortex	
Sham	--	28 ± 2.3	61 ± 5.1	(6)[a]
Hypox	--	27 ± 1.7	30 ± 1.1	(9)
Hypox	10 IU	26 ± 1.8	157 ± 25.3	(6)[a]
Hypox	20 IU	28 ± 1.5	193 ± 52.3	(3)[a]

Values shown are mean ± SEM (number of animals in parentheses). Five days after surgery, the rats were injected with control solution and 10 or 20 IU ACTH. Animals were killed 12 h after.

[a] $P < 0.001$ for comparison of indicated mean with hypophysectomized controls.

TABLE III Effect of apomorphine on adrenomedullary ODC activity in rats after transection of the spinal cord

Treatment	Control		Drug-treated		P
Sham-operated	28 ± 8.2	(6)	620 ± 117.7	(6)	<0.001
Sectioned at T_5	81 ± 21	(7)[a]	249 ± 42.7	(7)[a]	<0.001
Sham-operated	18 ± 5	(6)	1406 ± 238	(4)	<0.001
Sectioned at T_2	24 ± 6	(6)	117 ± 38.9	(5)[b]	<0.01

Values shown are mean ± SEM (number of animals in parentheses). For other experimental details see legent to Table I.

[a] $P < 0.01$ for comparison of indicated mean with sham-operated rats

[b] $P < 0.001$ for comparison of indicated mean with sham-operated rats

TABLE IV Effect of intracerebroventricular injection of 6-hydroxydopamine

Treatment	Control		APM-treated		P
-----	25 ± 1.8	(6)	553 ± 133.5	(6)	< 0.001
6-OHDA	39 ± 10	(7)	830 ± 180.0	(6)	< 0.001

Animals were injected into the right lateral ventricle with 125 µg/rat 6-OHDA one hour after the pretreatment with 20 mg/kg i.p. desipramine. Controls were injected with carrier solution and desipramine. One week after, they were given three doses of APM (10 mg/kg s.c.) at 0, 1.5 and 3 h and were decapitated 4 h after the first injection of the drug. P values indicate the level of significance for the difference between control and treated groups.

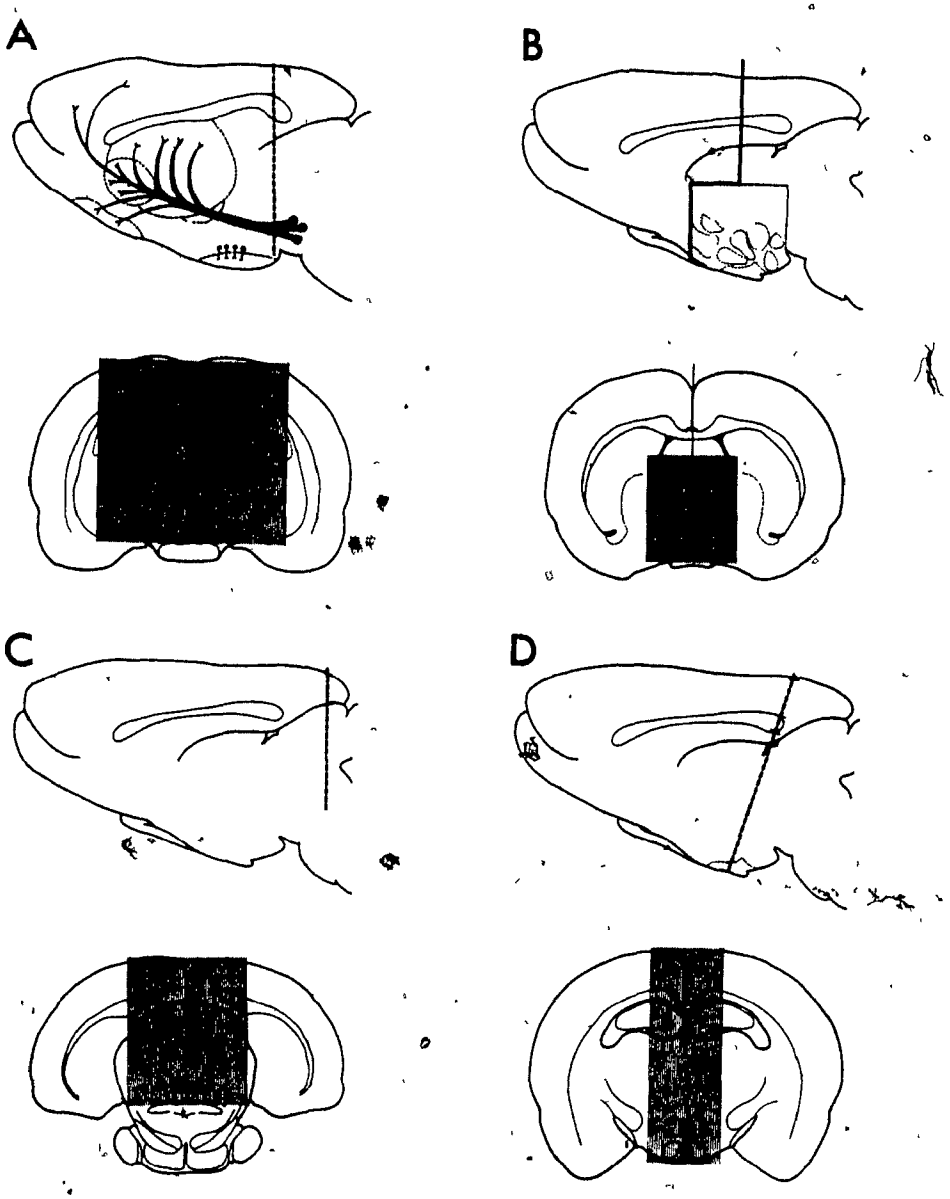

FIGURE 4

Schematic diagram of the sagittal and coronal views representing: A, diencephalic transection (main dopaminergic pathways on sagittal view); B, hypothalamic island; C, incomplete mesencephalic transection; and D, incomplete diencephalic transection.

TABLE V Effect of apomorphine on adrenomedullary ODC activity in rats after section of the brain at various levels

Lesion	Control		Drug-treated		P
Sham	46 ± 6.3	(6)	255 ± 29.3	(7)	< 0.001
Mesencephalic	46[d] ± 6.3	(6)	286 ± 28.2	(6)	< 0.001
Diencephalic	21 ± 3.2	(4)	66 ± 12.6	(6)[c]	< 0.01
Hypothalamic island	46 ± 6.5	(5)	481 ± 63.7	(6)[a]	< 0.001
Incomplete diencephalic	49 ± 15.2	(5)	427 ± 31.2	(7)[b]	< 0.001

Values shown are mean ± SEM (number of animals in parentheses). 24 h after the surgery, the rats were injected with APM according to Table I.

[a] $P < 0.05$ for comparison of indicated mean with sham-operated rats

[b] $P < 0.01$ for comparison of indicated mean with sham-operated rats

[c] $P < 0.001$ for comparison of indicated mean with sham-operated rats

[d] Results pooled with those obtained in sham-operated controls (see text).

P values indicate the level of significance for the difference between control and treated groups.

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CHAPTER VI

Role of Serotonin in the Apomorphine-induced Increase of Adrenomedullary Ornithine Decarboxylase Activity

SUMMARY

The role of serotonin in the regulation of adrenomedullary ornithine decarboxylase (ODC) activity has been explored in rats after systemic administration of p-chlorophenylalanine (PCPA) and intraventricular 5,6-dihydroxytryptamine (DHT) or in animals with electrolytic lesions of the medial and dorsal raphe nuclei. None of the treatments produced any alteration in endogenous ODC activity. However, all except lesion of the dorsal raphe nucleus significantly potentiated the induction of adrenomedullary ODC produced by apomorphine (APM) administration. It is suggested that serotonergic fibers originating partly in the medial raphe nucleus exert a tonic inhibitory action over the APM-induced increase in adrenomedullary ODC activity.

INTRODUCTION

Ornithine decarboxylase (ODC, EC 4.1.1.17) is the rate-limiting enzyme in polyamine biosynthesis (37). This enzyme is present in many tissues of the body, including the adrenal glands. In the adrenal medulla, its activity can be reflexly induced by subjecting animals to cold exposure (12,33), or bodily restraint (32) for short periods, or by injecting them with reserpine (16,32,34), cholinergic agonists (33,34), or dopaminergic agents (1,2,3). Work in progress in this laboratory with rats bearing lesions transecting the brain at various levels has led us to conclude that the primary site of stimulation of dopamine-sensitive structures by apomorphine (APM) is at the level of the diencephalon-telencephalon. We have also observed that after transection of the brain at the level of the posterior hypothalamus through a lesion that extends laterally 1.5-2 mm to each side of the midline, the effect of APM on the induction of adrenomedullary activity of ODC is potentiated. Because this lesion must sever the medial ascending serotonergic pathways, there is the possibility that a serotonergic system serves in the regulation of ODC, through exerting a tonic inhibitory action relieved by the lesion.

There is already much histological and pharmacological evidence for the existence of functional interactions between serotonergic and dopaminergic systems in the brain (29). Of special pertinence to the present work are the findings that tonic inhibitory impulses originating in the medial raphe nucleus (MRN) are important in the regulation of the

activity of another inducible adrenomedullary enzyme, tyrosine hydroxylase, and that these impulses function together with an excitatory influence from a dopaminergic center in the brain (30,31). Therefore, in attempting to define the role of serotonin in the regulation of adrenomedullary ODC activity we have investigated the effects of specific depletors, p-chlorophenylalanine and 5,6-dihydroxytryptamine, and of selective electrolytic lesions of the dorsal and medial raphe nuclei on the activity of this enzyme. To study the possible interaction of serotonin with dopamine, these treatments have been used in combination with APM.

MATERIALS AND METHODS

Materials

Apomorphine hydrochloride was purchased from F.E. Cornell and Co., Montreal, Quebec. Pyridoxal 5'-phosphate, DL-dithiothreitol, 5,6-dihydroxytryptamine creatinine sulfate (DHT), 6-hydroxydopamine hydrobromide (6-OHDA), and DL-p-chlorophenylalanine methyl ester hydrochloride (PCPA) were obtained from Sigma Chemical Co., St. Louis, MO. L-(1-^{14}C) Ornithine, specific activity 40-60 Ci/mol, and 2,5-diphenyloxazole were purchased from New England Nuclear, Boston, MA. All other chemicals were obtained from standard commercial sources.

Animals

Male adult Sprague-Dawley rats were used throughout this work. Weights of particular groups are specified below, except for intact (non-operated rats) which weighed 203 g ± 1.9 (SEM). The animals were

obtained from Canadian Breeding Farms and Laboratories Ltd., St. Constant, Quebec. They were kept in the animal room in individual wire cages under a light-dark cycle of 12:12 h with tap water and Purina Checkers ad libitum.

Apomorphine was dissolved, 3.3 mg/ml, in 0.1% solution of sodium metabisulfite to prevent oxidation. It was injected (10 mg/kg i.p.) on three occasions at 0, 1.5 and 3 h. Animals were killed 4 h after the initial injection. It has been previously shown that a single dose of APM does not affect ODC activity up to 4 h after its administration. At least one additional injection of the drug is necessary to increase ODC activity. In this work, we have used a standard treatment of three doses (1).

PCPA, 50 mg/ml, was dissolved in 0.9% saline; it was injected intraperitoneally in two doses of 150 mg/kg, 24 h and 12 h before APM. Both APM and PCPA were titrated with 0.1 N NaOH to arounf pH 6 and injected in a volume of 3 ml/kg.

DHT and 6-OHDA were dissolved in 0.9% saline containing ascorbic acid, 1 mg/ml, and were injected into the right lateral ventricle one week before APM in a volume of 10 µl (containing 75 µg DHT or 125 µg 6-OHDA) over a two-min period.

Surgical procedures

All surgery was performed in this laboratory under chloral hydrate anesthesia (300 mg/kg i.p.), with rats in a stereotaxic apparatus. The coordinates of de Groot (17) were used for the intraventricular injection

of the neurotoxins, and those of König and Klippel (25) for introducing electrolytic lesions. Intracerebral injection of DHT and 6-OHDA was carried out in rats weighing 243 g ± 2.4. A Hamilton microsyringe was used. Sham-operated controls were injected with the same volume (10 µl) of carrier solution. All brains were examined postmortem to check the trajectory of the needle into the ventricle. Electrolytic lesions of the raphe nuclei were carried out in rats weighing 246 g ± 3.2. A burr hole was made in the midline over the midbrain raphe nuclei. Anodal electrolytic lesions were produced by passing a current of 2 mA for 30 sec (MRN) and 1.5 mA (dorsal raphe nucleus, DRN) through a stainless steel needle (25-gauge) insulated but for 0.7 mm at the tip. A large electrode clipped to the skin of the head was connected to the cathode. Stereotaxic coordinates (25) were A 0, L 0 and either V -1.5 (DRN) or V -3 (MRN). Sham-operated control animals were treated in the same manner except that no current was passed after the electrode had been lowered intracranially. These surgical procedures have been previously employed in rats, and the resulting changes in the serotonin content of the brain have been reported after electrolytic lesions of the raphe (31) and administration of DHT (30). Histological control of the lesions has been done in this work as before (31) so that animals with lesions lying outside the dorsal or medial raphe nucleus were not included in the calculations of experimental results. The changes in brain catecholamines after intraventricular injection of 6-OHDA were

also reported previously (30).

Tissue preparation

Upon decapitation of the animals the adrenals were quickly removed and dissected at 4°. Contamination of medullary tissue by cortex was estimated to be about 15%; this was based upon the measurement of corticosteroids present in both portions of the dissected tissue by a microfluorometric method as previously described (1).

The portion of the tissue corresponding to two medullae was pooled and homogenized in 200 µl of sodium-potassium phosphate buffer, 0.05 M, pH 6.8 with motor-driven Teflon pestle. The homogenate was centrifuged at 20,000 g for 20 min, and the supernatant portion was taken for ODC assay.

ODC assay

Determination of ODC activity with L-(1-^{14}C)ornithine as substrate, was performed as previously described (1,32). The reaction mixture contained 1 µCi of L-(1-^{14}C)ornithine, 0.04 mM; pyridoxal 5'-phosphate, 0.05 mM; dithiothreitol, 0.01 mM; EDTA, 0.1 mM; and 100 µl of enzyme preparation in 0.5 ml final volume. The activity of the enzyme is always expressed as pmol $^{14}CO_2$ produced per mg protein per 45 min incubation at 37°.

Statistical procedures

Data in the tables and figures are represented as mean ± standard error of the mean (SEM). As the variance tended to vary directly with

the size of the mean, individual values were transformed logarithmically prior to calculation of statistics used in assessing the significance of the data, i.e. Student's t-test in comparing two means and Fisher's F in the anaylsis of variance (36).

RESULTS

Effect of PCPA on adrenomedullary ODC activity

The systemic administration of PCPA irreversibly inhibits tryptophan hydroxylase, the rate-limiting enzyme in 5HT synthesis (23), and brings about a severe depletion of serotonin content in the brains of mice, rats and dogs (24). In our experiments, PCPA was administered intraperitoneally in two doses of 150 mg/kg each at 24 h and 12 h before the administration of APM. This dosage reduces brain serotonin to 15% of control (11). PCPA given alone had no significant effect on the resting medullary ODC activity as compared to controls injected with inert vehicle (Table I). APM (three doses of 10 mg/kg i.p.) produced a large increase in ODC activity, viz. 15-fold over control activity, as previously observed in this laboratory. The administration of APM to rats pretreated with PCPA showed much larger increases, amounting to an additional 300% potentiation (Table I).

Effect of 5,6-dihydroxytryptamine and 6-hydroxydopamine

The neurotoxin DHT given intraventricularly effects a long-lasting and selective depletion of brain and spinal cord serotonin (6). This drug acts by causing degeneration of nerve axons and terminals (7).

Further evidence of its neurotoxic action on serotonergic fibers is demonstrated by the reduction in brain tryptophan hydroxylase (10). In this work, DHT was administered to rats into the right lateral ventricle, and 7-10 days later APM was given as previously described. Animals treated with DHT alone had no change in adrenomedullary ODC activity. As with PCPA, rats treated with DHT and APM showed larger increases in enzymic activity than after the administration of APM alone ($P < 0.01$). In this case the potentiation of the effect of APM by DHT was doubled (Table I).

To check if the effect of DHT is selectively due to a decrease in serotonin content and not to damage to catecholaminergic neurons, a group of rats was injected intraventricularly with 6-OHDA. This neurotoxic amine causes large decreases in brain noradrenaline and dopamine (9,30). 6-OHDA alone, as was the case with DHT, did not have any significant effect on adrenomedullary ODC activity of controls. Furthermore, when given in combination with APM, it did not cause a potentiation (Table I, Line 3).

The results obtained with PCPA and DHT strongly suggest that a central serotonergic system exerts an inhibitory role over the APM-induced increase in adrenomedullary ODC activity.

Effects of electrolytic lesions of dorsal and medial raphe

Because dorsal and medial raphe nuclei contain a large number of the 5HT cell bodies whose axons project to the forebrain (5,14), and because of the evidence for direct 5HT projections from both nuclei to

dopaminergic centers (13,15,18,27), discrete electrolytic lesions of the individual nuclei were made.

The position of the electrolytic lesion was confirmed histologically in each animal by examining serial coronal sections through the midbrain (Figure 1) under the dissecting microscope. The brains had been fixed for two weeks in 10% formaldehyde. If the lesions had been inaccurately placed, the results were discarded.

Seven to ten days after lesion of either DRN or MRN there was no significant difference in resting adrenomedullary ODC concentration, by comparison with sham-operated controls (Table II). This suggests that neither of the affected raphe nuclei plays a significant role in the maintenance of steady-state levels of adrenomedullary ODC activity.

Administration of APM caused large increases of ODC activity in all groups studied: sham-operated rats, and those with lesion of either the DRN or the MRN (Table II). In addition, there was some potentiation of the effect of APM in animals bearing lesion of the MRN ($P < 0.05$).

DISCUSSION

In this study we examined the possible participation of a serotonergic system in the regulation of adrenomedullary ODC activity, both the resting (endogenous) activity and that induced by treatment of the animals with APM. The use of depletors of cerebral serotonin, i.e. PCPA and DHT, produced no significant changes in the control values of adrenomedullary ODC activity. This contrasts with the effects of these

compounds with respect to locomotor activity, which is increased (19,38), and adrenal tyrosine hydroxylase activity, which is also augmented (10,29).

In contrast to the failure of PCPA and DHT to influence the resting level of adrenomedullary ODC activity, both drugs produced clear potentiation of the effect of APM in inducing that enzyme (Table I).

The effect of DHT on serotonergic neurons has been found to be selective because administration of 6-OHDA which destroys both dopaminergic and noradrenergic neurons did not potentiate the effect of APM on adrenomedullary ODC activity.

Numerous anatomical and electrophysiological studies give support for the existence of functional interactions between 5HT and DA systems in the brain. Thus, serotonergic projections have been demonstrated as follows: from the DRN to the substantia nigra and striatum by Fibiger and Miller (20); from the DRN to neostriatum, and from the substantia nigra to DRN by Pasquier et al. (27); from DRN to caudate-putamen by Miller et al. (29); and from MRN to substantia nigra by Dray et al. (18) and Bobillier et al. (8). Separate electrolytic lesions of the DRN and MRN were made in an effort to define the possible participation of a specific serotonergic center in the potentiation on the APM effect on adrenomedullary ODC activity. Neither lesion produced an alteration in the control values in comparison to sham-operated controls, a result consistent with the previously noted lack of serotonin depletors (Table I). On the other hand, destruction of the MRN produced a

potentiation of response of adrenomedullary ODC to APM. This indicates that the MRN exerts an inhibitory influence over the APM-induced increase in enzymic activity, an effect that is not shared by the DRN (Table II). This inhibitory role of fibers emanating from the MRN has previously been detected in regard to the induction of adrenal tyrosine hydroxylase (31), as well as to motor activity in rats (22). Both induction of adrenal tyrosine hydroxylase and motor hyperactivity occur spontaneously after production of the lesion, in contrast to adrenomedullary ODC activity.

Three treatments were tested in the attempt to influence the serotonergic mechanisms that are concerned with induction of adrenomedullary ODC. These were, in order of their decreasing potency, systemically administered PCPA, intraventricularly injected DHT, and electrolytic lesion of the median raphe nucleus. The last treatment affects only a portion of the serotonergic ascending pathways in the brain (14,28). The relative effect of DHT in depleting serotonin stores in the brain is limited by its ability to diffuse into the parenchyma from the ventricles and thereby to reach distant serotonergic axons (35). By contrast to these treatments, systemically injected PCPA readily crosses the blood-brain barrier and causes a fairly uniform reduction of serotonin content in various parts of the brain through the reduction of tryptophan hydroxylase activity. Our results, therefore, do not exclude the involvement of other serotonergic centers in this regulation.

Thus, descending spinal serotonergic projections (26), with an inhibitory component, may be implicated in the regulation of adrenal tyrosine hydroxylase activity (21) and in the insulin-induced depletion of adrenal adrenaline (4).

In conclusion, experiments with DHT, PCPA and electrolytic lesion of the raphe nuclei suggest that a serotonergic pathway (or pathways) originating in the MRN exerts an inhibitory (braking) influence over the induction of adrenomedullary ODC activity, at least as elicited by APM.

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FIGURE 1

Coronal view of a typical electrolytic lesion to: I, medial raphe; and II, dorsal raphe nucleus.

TABLE I. Adrenomedullary ODC activity after p-chlorophenylalanine (PCPA), 5,6-dihydroxytryptamine (DHT) or 6-hydroxydopamine (6-OHDA) with or without apomorphine (APM)

PCPA (300 mg/kg i.p.) was injected in two doses of 150 mg/kg, 24 h and 12 h before the administration of APM (3 doses of 10 mg/kg at 0, 1.5 and 3 h). DHT (75 µg/rat) or 6-OHDA (125 µg/rat) was injected into the right lateral ventricle, one week before APM. Animals were killed 4 h after the first injection of APM in all cases. Figures are given as pmol $^{14}CO_2$ per mg protein per 45 min and represent mean ± SEM for the number of rats in parentheses. The P value indicates the level of significance for the difference between control and APM-treated groups.

[a] $P < 0.001$, [b] $P < 0.01$ for comparison of indicated mean with rats treated with APM only.

Treatment	Control		APM-treated		P
-----	95 ± 7	(7)	647 ± 51	(5)	< 0.001
DHT	116 ± 19	(5)	1039 ± 83	(5)[b]	< 0.001
6-OHDA	63 ± 13	(5)	691 ± 204	(4)	< 0.01
-----	27 ± 5	(6)	392 ± 56	(6)	< 0.001
PCPA	37 ± 4	(6)	1304 ± 123	(7)[a]	< 0.001

TABLE II. Effect of apomorphine (APM) on adrenomedullary ODC activity in rats with electrolytic lesion of the raphe nuclei

APM was injected i.p. in three doses of 10 mg/kg at (0, 1.5 and 3 h) 7-10 days after electrolytic lesions of the raphe nuclei or sham-operation. Figures are given as pmol $^{14}CO_2$ per mg protein per 45 min and represent mean ± SEM for the number of rats in parentheses. The P value indicates the level of significance for the difference between the control and APM-treated groups.

[a] $P < 0.05$ for comparison of indicated mean with sham-treated with APM.

Treatment	Control		APM-treated		P
Sham	59 ± 6	(6)	269 ± 55	(6)	< 0.001
Lesion of dorsal raphe	48 ± 3	(6)	209 ± 30	(7)	< 0.001
Sham	64 ± 6	(7)	288 ± 38	(9)	< 0.001
Lesion of medial raphe	82 ± 12	(8)	424 ± 44[a]	(10)	< 0.001

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CHAPTER VII

Neurohumoral Regulation of Adrenocortical Ornithine Decarboxylase Activity: Dopaminergic-Serotonergic Interactions

SUMMARY

Administration of the dopamine receptor agonists apomorphine (APM) and piribedil increase adrenocortical ornithine decarboxylase (ODC) activity. In this paper alterations in the activities of the sympathoadrenal system and of the central dopaminergic and serotonergic systems have been produced to study the possible mechanism of action of APM on adrenocortical ODC. Unilateral splanchnicotomy and rhizotomy, and bilateral demedullation attenuated the response of adrenocortical ODC to APM. Intraventricular 6-hydroxydopamine and 5,6-dihydroxytryptamine, intraperitoneal p-chlorophenylalanine and electrolytic lesion of the medial raphe nucleus reduced the APM-induced increase. None of these treatments produced any statistically significant changes in the endogenous ODC activity of the adrenal cortex. It is postulated that dopaminergic brain structures participate directly in the stimulatory effect on the hypophyseal-adrenal system to increase adrenocortical ODC activity. An intact central serotonergic system seems to be necessary for APM to exert its effect on adrenocortical activity, particularly the medial raphe nucleus.

INTRODUCTION

Ornithine decarboxylase (ODC, EC 4.1.1.17) catalyzes the conversion of ornithine to the diamine putrescine, in a reaction that appears to be rate-limiting for the biosynthesis of polyamines (48). The enzyme is found in many tissues, where it may be induced when the animal is subjected to a variety of stimuli, such as the administration of certain hormones or drugs, or is placed under stress even for short periods of time (30,39,46). Thus, the muscarinic agonist oxotremorine (36), and dopaminergic agents (1,2,3) provoke the induction of adrenocortical ODC activity. Haloperidol, a dopamine antagonist, prevents the action of apomorphine (APM) in the first four hours after the administration of that dopaminergic drug, but by the sixth hour the antagonism is not as evident, for by that time haloperidol itself has caused a significant increase in adrenocortical ODC activity (2,3). The effects of oxotremorine and the dopaminergic drugs APM and piribedil are abolished in hypophysectomized rats; this demonstrates that their actions in regard to induction of adrenocortical ODC are ultimately mediated by the pituitary gland.

Previous work on the mechanism of action of APM on adrenocortical ODC activity has now been extended. The action of the drug has been explored in rats after denervation of the adrenal glands, adrenal demedullation, and chemical denervation of central dopaminergic systems. In addition, the possible participation of a serotonergic system in the regulation of adrenocortical ODC activity has been investigated by taking measures to alter brain serotonin levels in rats.

MATERIALS AND METHODS

The materials, tissue preparation, ODC assay and statistical procedures are the same as in Chapters II, IV and V. All surgical procedures have already been described also, except for adrenal demedullation.

Bilateral demedullation in rats weighing 153 g ± 2 was carried out after midline laparotomy. Three weeks after the operation, animals weighed 300-315 g whereas sham-operated controls weighed 250-265 g. Operated animals received 0.9% sodium chloride solution instead of drinking water. Examination of the tissue at the end of the experiment showed that about 70% of the operated rats were totally enucleated. Measurement of catecholamines by HPLC in demedullated adrenals showed that the amount of medullary tissue remaining in the glands of the other 30% was 5% or less than in intact adrenals.

RESULTS

Effect of apomorphine on adrenocortical ODC activity

The control levels of ODC in the adrenal cortex of the rats can be increased 3-10 times by multiple injections (2-4) of APM, 10 mg/kg i.p. The time-course of the effect was recently studied with two doses of APM (2). As is clear in Table I, the effect of APM on adrenocortical ODC activity is dose-related, at least two doses being necessary to increase the enzyme activity significantly over control values. Moreover, at 6 h the response of adrenocortical ODC is larger than at 4 h. That is, two doses produced an increase of 191% over controls at 4 h, but 336% at 6 h.

With three doses of APM, the corresponding increases were 401% and 756%.

In subsequent work three doses of APM were used, and animals were killed at 4 h after the first dose. This schedule was chosen to accommodate experiments on ODC activity of the adrenal medullae of the same animals (1,2). Although the cortical response at this time-point has not yet attained its maximum, both structures of the adrenal show large and significant responses to the APM treatment.

Effect of splanchnicotomy on the APM-induced increase

Table II shows that the mean endogenous adrenocortical ODC activity of the denervated gland is slightly higher than the intact, but this difference is not statistically significant. In animals given APM, there was a rise in adrenocortical ODC values over the controls on both sides. The apparent higher increase in the intact side compared to the denervated side produced by APM can be attributed to contamination by the highly responsive medullary tissue: medullary ODC of the intact side amounted to 763 ± 63 pmol $^{14}CO_2$ per mg protein per 45 min as compared with 60 ± 5.9 for the denervated side. When cortical values are corrected for 10% contamination by medullary tissues (see Methods) with these respective activities, the real increases produced by APM are no longer as large as in Table II, but nevertheless amount to a doubling of control values on both sides. When the values obtained with APM in unilaterally splanchnicotomized animals (Table II, line 2) are compared to those in intact rats (Table I, line 3), or sham-operated (Table III, line 3), splanchnicotomy appears to attenuate the effect of APM on ODC activity of the adrenal cortex, and this holds even after correction for presence of

medullary fragments.

Effect of rhizotomy

To investigate further autonomic effects on the ODC activity of the adrenal cortex, some rats underwent unilateral rhizotomy, that is, section of ventral or dorsal spinal roots or a combination of these. Five days after surgery, they were injected with three doses of 10 mg/kg APM. Rhizotomy, independently of the number of roots sectioned (1-4) and of the region studied (T_4-T_{10}), produced an attenuation of the APM-effect on adrenocortical ODC activity of both intact and operated sides as compared to control rats treated with the drug (Table I). Moreover, this attenuation occurred after section of either ventral or dorsal roots, or both, and the adrenocortical response on the two sides was essentially equal.

Effect of demedullation

To check whether the response of the cortical enzyme is in any way dependent upon the presence of the adrenal medulla, some rats were bilaterally demedullated. They were used for the drug experiment three weeks after the surgery to allow for complete regeneration of the cortical tissue, as ODC activity is elevated during the early regeneration process (31). Basal levels of ODC activity of demedullated adrenals (Table III) were comparable to cortical enzyme activity in sham-operated rats. Administration of APM brought about a significant increase in ODC activity over controls at both 4 h and 7 h, an approximate doubling in each case. However, the ODC response of demedullated adrenals was much lower than in adrenal cortex of the sham-operated or non-operated rats (2-fold vs 5-6-fold).

The second important difference in the response of adrenocortical ODC to APM in rats with demedullated adrenals as compared to intact rats is that this increase in enzymic activity is not time-dependent. APM doubles the adrenocortical ODC activity over controls at both 4 and 7 h in rats with demedullated adrenals (Table III). On the other hand, the same dose of the drug causes a 5-fold increase at 4 h and an 8.5-fold increase at 6 h in intact rats (Table I, lines 3 and 7). Contamination of the adrenocortical tissue by medullary tissue is not responsible for these differences. In fact, if all the values are corrected by the 10% estimated contamination (see Methods) the increment in activity is similar to uncorrected results (see legend to Table I for corrected values).

Effect of intracerebroventricular 6-hydroxydopamine

Since the demonstration by Thoenen and Tranzer (49) of the destruction of catecholamine-containing nerve terminals by 6-OHDA, the injection of neurotoxins into brain of experimental animals has been used to investigate the role of aminergic systems in various physiological, pharmacological and behavioral responses. 6-Hydroxydopamine was used in the present experiments by injecting it into the right lateral ventricle. This is known to cause decreases in cerebral catecholamine levels 24 h after injection (50). To render the action of the drug more specific for dopamine, the rats were treated with desipramine, which blocks the uptake of the drug by noradrenergic, but not dopaminergic, neurons (8). APM (10 mg/kg i.p., three doses) was administered to rats 6-8 days after injection of 6-OHDA.

Rats treated in this way showed a reduction of 64% in the response of adrenocortical ODC activity to APM (Table IV, corrected values). It should be noted that the control animals, injected with the vehicle intraventricularly and therefore subjected to the same surgical stress as the others, showed normal increases in adrenocortical ODC activity after APM. Hence, the effect of the neurotoxin cannot be attributed to a reduction in response owing to this invasive technique.

Intraventricular administration of 5,6-dihydroxytryptamine

Central serotonergic mechanisms antagonize amphetamine-induced hyperactivity in rats (9) and APM-induced increases in adrenal tyrosine hydroxylase activity (34). In seeking a possible dopaminergic-serotonergic relationship in the control of adrenocortical ODC activity, rats were injected intraventricularly with the neurotoxic tryptamine derivative 5,6-dihydroxytryptamine (DHT). This drug causes long lasting selective depletion of brain serotonin (4). In our experiments, DHT alone had no effect on adrenocortical ODC activity. However, when the drug was administered to rats in combination with APM, it caused a reduction in the response of the enzyme to that drug (Table IV). The absolute values for cortex must be adjusted for an estimated 10% contamination by ODC of adrenomedullary origin. Because the medullary portion of the enzyme increases very highly in rats treated with both drugs, the precise correction is difficult to estimate, but it is clear that cortical ODC values, corrected for this contamination are extremely low in rats given DHT. Of course, even the uncorrected values for the adrenal cortex are

only 50% of those observed in sham-operated rats treated with APM (Table IV). Hence, it is clear that DHT has produced a profound decrease in the responsiveness to APM injection.

Effect of p-chlorophenylalanine

The administration of p-chlorophenylalanine (PCPA) brings about a severe depletion of serotonin content in the brain (24). In the present work, 300 mg/kg PCPA produced no alteration in the basal levels of ODC activity in the adrenal cortex, but it reduced the uncorrected response of adrenocortical ODC to APM by about 33%, and the corrected values by 80% (Table IV). These data, as well as those with DHT, show a clear tendency towards inhibition or abolition of APM-induced increase of adrenocortical ODC activity.

Effect of electrolytic lesions of the dorsal and medial raphe nuclei

The results obtained with DHT and PCPA demonstrate that the serotonin content of the brain modulates the response of adrenocortical ODC to APM. Electrolytic lesions of the separate mesencephalic raphe nuclei were carried out, in an effort to define the possible participation of specific serotonergic centers in this effect. The results are shown in Table V. Lesion of the dorsal raphe produced no alteration in the response of adrenocortical ODC to APM, and only a small reduction (23%) was observed after lesion of the median raphe. However, this reduction was statistically significant in comparison with sham-operated rats treated with the drug.

DISCUSSION

As previously shown, the administration of APM to rats causes a large

increase in adrenocortical ODC activity. This increase is time-dependent and specifically blocked at 4 h by the pretreatment with the dopamine receptor antagonist haloperidol (2). Our present results demonstrate that the effect of APM on adrenocortical ODC is dose-related, and also that, for a given schedule of injections, cortical ODC attains higher levels of activity at 6 h than at 4 h after the start of that schedule.

The mechanism of action of APM on adrenocortical ODC activity must be due to the release of pituitary factors.

Three anterior pituitary hormones are known to stimulate adrenal ODC in rats: ACTH (27,28,38), growth hormone (27), prolactin (37,51). It seems unlikely that the effect of APM on adrenocortical ODC could be mediated by release of either of the last two factors. In the first place, administration of APM to rats reduces the concentration of plasma prolactin (10) and decreases prolactin secretion in anterior pituitaries of rat in vitro (44). Secondly, although APM causes a prompt increase in plasma growth hormone in humans (26) it has little or no effect in male rats (25). On the other hand, APM administration to rats increases the levels of circulating corticosterone reflecting an increased rate of release of ACTH from the pituitary (17,25). Release of other pituitary peptides such as endorphins or opiates is also possible. Both have been reported to stimulate corticosterone synthesis in vitro (19,45).

Although the adrenocortical increase produced by APM is ultimately mediated by the pituitary (2), just as in the case of oxotremorine (36), a potential role of adrenal innervation in augmentation of the activity

has been suggested. For this reason, rats were unilaterally splanchnicotomized. In control rats, ODC activity was slightly higher in the denervated adrenal, but APM administration produced a similar increase in both glands. It has been shown that under normal conditions the denervated glands shows hyperactivity (12,41,47,52). That is, splanchnicotomy results in an increased adrenal weight as compared to sham-operated controls, and in slightly higher levels of corticosterone. However, the stress of immobilization produces similar response in both intact and denervated adrenals (52). In our experiments, we have found that the adrenocortical ODC response to APM is attenuated, and this is true for both intact and denervated adrenals. Furthermore, unilateral section of ventral or dorsal spinal cord roots or combined dorsal and ventral roots all produce a similar effect: bilateral attenuation of the APM-induced increase in adrenocortical ODC activity. These results with unilateral splanchnicotomy and rhizotomy suggest that the adrenocortical ODC response to APM stimulation is not directly dependent on the nerve supply to the adrenal medulla. However, a reflex mechanism appears to modulate this regulatory function. As the attenuation of the APM effect is observed in both glands, at least the efferent component (i.e. directed towards the adrenal gland) must be humoral. The afferent limb of the reflex arc could be either nervous or humoral.

Because adrenomedullary ODC activity is increased by the administration of APM, and responds earlier (2 h as compared to 4 h for the cortex) (2), the possible mediation of the adrenal medulla in the cortical increase produced by APM was investigated in the following way: APM was administered

three weeks after bilateral demedullation. Although its effect was decreased in such rats it still caused a significant increase in controls (Table III). This increase did not display the time-dependency noted for adrenocortical ODC activity of intact rats (Table I). Thus, the data demonstrate that, although APM can cause a significant increase in adrenocortical ODC activity without benefit of the medulla, the medulla must play an important role for the time-dependent increases observed in intact rats. It is possible that the effect of APM on intact or sham-operated rats is partly regulated by a reflex mechanism operating through the adrenal medulla; the mechanism would entail excitation of dopaminergic centers in the brain with sympathetic effects in the periphery, occasioned by the release of adrenal epinephrine. The changes in the periphery would, in turn, induce reflex stimulation of the pituitary-adrenal complex. The pituitary gland itself responds to both humoral and neural inputs (20); and adrenal steroids are known to exert both fast and delayed feedback at the level of the hypothalamus and the anterior pituitary (22,42). In the case of adrenal demedullation, the levels of circulating steroids are also altered (6).

From our data, we cannot decide whether the attenuation of the adrenocortical ODC response to APM is due to: (i) elimination of a facilitatory feedback arising from the medulla; (ii) the action of an inhibitory feedback arising from the cortex itself; or (iii) a combination of the two. We can only conclude that the physiological changes either neural and/or humoral, caused by the surgical manipulation (splanchnicotomy,

spinal root sections and demedullation) exert some kind of modulatory effect on the APM-induced increase in adrenocortical ODC activity.

Chemical denervation of central dopaminergic fibers with 6-OHDA produced a considerable reduction in the APM-induced increase in adrenocortical ODC activity. These results suggest that dopaminergic brain structures participate directly in the stimulatory effects on the pituitary-adrenal system. Indeed, there are dopamine-containing fibers in the median eminence that originate from cell bodies in the arcuate and ventral anterior periventricular nucleus of the hypothalamus (18). In addition, a portion of the dopaminergic fibers of the median eminence originate from neurons in the substantia nigra (23). Interestingly, Cuello, Weiner and Ganong (11) have described resistance of the hypothalamic dopaminergic neurons to the depleting effects of high doses of 6-OHDA in the rat. However, our experiments do not implicate particular pathways, so that further studies would be necessary to locate specific dopaminergic centers responsible for the APM-induced increase in adrenocortical ODC activity.

The possible participation of a serotonergic system in the regulation of adrenocortical ODC activity has also been investigated by treatment of rats with PCPA to inhibit tryptophan hydroxylase, and intracerebroventricular injection of DHT to destroy serotonergic nerve terminals. Neither produced an alteration in the control values of adrenocortical ODC activity, but both treatments reduced the response to APM. These data might indicate that serotonin plays a tonic facilitatory role in the response.

Electrolytic lesions of the dorsal raphe nucleus failed to mimic the action of PCPA or DHT. However, destruction of the medial raphe produced

a small but significant reduction in the response of adrenocortical ODC to APM. This result suggests that the medial, but not the dorsal, raphe nucleus plays some role in mediation of the APM effect.

The functional interaction between serotonergic and dopaminergic systems in the brain is well documented (7,9,14,15,29,33,34,35); and serotonergic innervation of the hypothalamus of intra- and extrahypothalamic origin has been described (5,16,18,13,32,40,53). It is particularly interesting to mention again that the serotonergic influence on the APM-induced increase in ODC activity is inhibitory for the medullary enzyme (1), but appears facilitatory for the adrenal cortex; although, both involve the medial raphe nucleus.

In conclusion, our experiments demonstrate that dopaminergic brain structures participate directly in the stimulatory effect on the hypothalamo-hypophyseal-adrenal complex to increase adrenocortical ODC activity. In addition, APM administration seems to promote a complex reflex mechanism entailing a flow of information pertinent to the regulation of adrenocortical ODC activity back to the brain by humoral or neural messages. This latter effect is currently being investigated in our laboratory. Furthermore, intact function of the medial raphe nucleus of the mesencephalon is necessary for APM to exert its effect on adrenocortical ODC activity.

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TABLE I Effect of apomorphine (APM) on adrenocortical ODC activity in intact rats

APM was administered in a dose of 10 mg/kg i.p., at 0 time, and rats were killed 4 or 6 h later. When more than one injection was used, the above dose was repeated at 1.5 h, 3 h and 5.5 h as indicated in the Table. Figures are given as pmol $^{14}CO_2$ per mg protein per 45 min and represent mean ± SEM for the number of observations in parentheses.

The significance of differences from controls was: [a] $P < 0.001$; [b] $P < 0.01$; [c] $P < 0.01$.

[d] This mean value, corrected for approximately 10% contamination with medullary tissue, becomes 63 pmol $^{14}CO_2$ per mg protein per 45 min. Corresponding drug-treated group, 307. The corrected means for these treatments at 6 h are 50 and 414, respectively.

Drug	No. of doses	Time	Control		Drug-treated		Percent of control
APM	1	4 h	35 ± 9.7	(3)	34 ± 15	(3)	97
APM	2	4 h	78 ± 25.9	(4)	227 ± 38.1	(7)[c]	291
APM	3	4 h	67 ± 14.6[d]	(4)	338 ± 57.4	(8)[b]	504
APM	1	6 h	86 ± 26.2	(3)	91 ± 40	(3)	106
APM	2	6 h	39 ± 7.3	(7)	170 ± 24.7	(8)[a]	436
APM	3	6 h	52 ± 12.3	(4)	445 ± 73.9	(6)[b]	856
APM	4	6 h	74 ± 6.7	(6)	817 ± 92.7	(9)[a]	1104

TABLE II Effect of apomorphine (APM) on adrenocortical ODC activity in rats after unilateral splanchnicotomy and rhizotomy

APM was administered i.p. or s.c. in a dose of 10 mg/kg at 1.5 h intervals (0, 1.5 and 3 h) 4-8 days after left unilateral splanchnicotomy or rhizotomy. Animals were killed 4 h after the first dose. Figures are given as pmol $^{14}CO_2$ per mg protein per 45 min and represent mean ± SEM for the number of observations in parentheses.

The significance of the differences from controls (non-drug-treated) were:

$^{a}P < 0.001$; $^{b}P < 0.01$; $^{c}P < 0.05$

Treatment		Drug	Denervated		Intact	
Splanchnicotomy		--	43 ± 10.2	(7)	30 ± 4.2	(7)
		APM	105 ± 16.1	(5)[b]	151 ± 17	(5)[a]
Rhizotomy						
T_{7-9}	V	APM	74 ± 6.9	(4)	71 ± 7.9	(4)
T_{7-9}	V	APM	151 ± 22.6	(5)[c]	135 ± 19.3	(5)[c]
T_{7-10}	D+V	APM	168 ± 55.8	(4)[c]	161 ± 24.5	(4)[c]
T_{4-6}	V	APM	135 ± 27.5	(5)[c]	152 ± 35	(5)[c]
T_8	V	APM	149 ± 38.9	(5)[c]	162 ± 35	(5)[c]
T_{7-10}	D	--	80 ± 6.9	(5)	77 ± 7.4	(5)
T_{7-10}	D	APM	177 ± 7.4	(5)[b]	193 ± 18.1	(5)[b]

TABLE III Effect of apomorphine (APM) on ODC activity of demedullated adrenals

Time	Control		Drug-treated		P
		Demdullated			
4 h	53 ± 7.8	(10)	120 ± 10.7	(11)	< 0.001
7 h	78 ± 8.6	(8)	153 ± 17.4	(9)	< 0.01
		Sham			
4 h	52 ± 6.6	(3)	298 ± 6.7[a]	(3)	< 0.001

Three weeks after bilateral demedullation, APM was administered to rats in a dose of 10 mg/kg s.c. at 0, 1.5 and 3 h. Animals were killed 4 h after the first injection of the drug. Figures are given as pmol $^{14}CO_2$ per mg protein per 45 min and represent mean ± SEM for the number of rats in parentheses. The P values indicates the level of significance for the difference between the control and treated groups.

[a] $P < 0.01$ for comparison of indicated mean with demedullated treated with APM.

TABLE IV Adrenocortical ODC activity after intracerebral injection of 6-hydroxydopamine (6-OHDA), 5,6-dihydroxytryptamine (DHT) and intraperitoneal p-chlorophenylalanine (PCPA), with and without apomorphine (APM).

6-OHDA (125 µg/rat) was injected into the right lateral ventricle of rats pretreated 1 h earlier with desipramine (20 mg/kg i.p.). DHT (75 µg/rat) was also injected into the right lateral ventricle. These neurotoxins were given 1 week before APM. PCPA (300 mg/kg i.p.) was injected in two doses of 150 mg/kg, 24 h and 12 h before the administration of APM (3 doses of 10 mg/kg at 0, 1.5 and 3 h). Animals were killed 4 h after the first injection of APM in all cases. Figures are given as pmol $^{14}CO_2$ per mg protein per 45 min and represent mean ± SEM for the number of rats in parentheses. The P value indicates the level of significance for the difference between control and treated groups.

[c] $P < 0.05$ for comparison of indicated mean with rats treated with APM only.

Treatment	Control		APM-treated		Corrected values	P
--	26 ± 3.0	(6)	287 ± 62.7	(6)	259	< 0.001
6-OHDA	30 ± 5.3	(7)	164 ± 38.3	(6)	90	< 0.001
--	66 ± 13.7	(7)	205 ± 38.6	(5)	159	< 0.001
DHT	56 ± 8.3	(5)	103 ± 22.5	(5)	0	< 0.001
--	29 ± 5	(6)	268 ± 30.4	(6)	226	< 0.001
PCPA	40 ± 4.3	(6)	180 ± 47	(7)[c]	50	< 0.001

TABLE V Effect of apomorphine (APM) on adrenocortical ODC activity in rats with electrolytic lesions of the dorsal (DR) and medial raphe (MR)

APM was injected s.c. in 3 doses of 10 mg/kg (at 0, 1.5 and 3 h) 7-10 days after electrolytic lesion of the raphe nuclei or sham-operation. Figures are given as pmol $^{14}CO_2$ per mg protein per 45 min and represent mean ± SEM for the number of rats in parentheses. The P value indicates the level of significance for the difference between the control and treated groups.

$^{a}P < 0.05$ for comparison of indicated mean with sham treated with APM.

Treatment	Control		APM-treated		Corrected values	P
Sham	126 ± 11.2	(6)	198 ± 20.4	(6)	171	< 0.05
DR lesion	116 ± 10.7	(6)	188 ± 24.5	(7)	164	< 0.05
Sham	127 ± 15.2	(7)	264 ± 15.5	(9)	235	< 0.001
MR lesion	131 ± 16.0	(8)	205 ± 25.6	(10)[a]	163	< 0.001

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CHAPTER VIII

Neurohumoral Regulation of Adrenocortical Ornithine Decarboxylase Activity

ABSTRACT

The increase in activity of adrenocortical ornithine decarboxylase (ODC) elicited by the administration of apomorphine (APM) was studied in rats, 4 days after transection of the spinal cord, or 24 h after various types of brain surgery: transection of the mesencephalon or of the diencephalon; hypothalamic deafferentation; or transection of the connections between hypothalamus and pituitary. Section of the cord elevated endogenous adrenocortical ODC activity and potentiated the induction by APM. Section at the level of the mesencephalon or interruption of the hypothalamic connections to the pituitary produced no change in either endogenous or induced ODC activity. In contrast, diencephalic transection produced a profound decrease in endogenous ODC and in response to APM. Hypothalamic deafferentation raised endogenous ODC concentrations and potentiated the response of the adrenal cortex to APM. The results strongly suggest that APM acts at the level of the diencephalon to increase adrenocortical ODC activity. However, diencephalic-mesencephalic connections must be intact for this to occur. Peripheral and extrahypothalamic influences play a modulatory role in this effect.

INTRODUCTION

Previous studies in our laboratory have shown that the activity of ornithine decarboxylase (ODC, EC 4.1.1.17) in the adrenal cortex of the rats is under the control of the pituitary gland. Hypophysectomy lowers the basal ODC levels and prevents the rise in adrenocortical ODC activity produced by oxotremorine (38) and by dopaminergic agonists (3) in intact rats. Moreover, the effect of one of these agonists, apomorphine (APM), is antagonized by the dopamine antagonist haloperidol (3). Several pituitary hormones are able to increase adrenal ODC in hypophysectomized rats: ACTH (29,30,42), growth hormone (29) and prolactin (41,51). The existence of neural regulatory mechanisms for the control of adenohypophyseal secretion is now well established (19). One of the neurotransmitters that has been identified with these regulatory systems is dopamine. It plays an important role in the secretion of prolactin, as demonstrated by neuropharmacological studies (5,25,34), and of growth hormone in humans (37). It may also serve in the regulation of ACTH (17,22,26). On these bases we decided to explore the role of various brain regions in the increase in adrenocortical ODC activity induced by the dopamine-receptor agonist APM. Male rats were surgically treated in order to produce gross separation of different brain regions. The animals were then injected with APM according to a schedule that invariably causes induction of ODC in intact rats. We also studied a possible somatic afferent influence on neuroendocrine control by subjecting some animals to transection of the spinal cord.

MATERIALS AND METHODS

The materials, tissue preparation, ODC assay, surgical and statistical procedures have been described in Chapters II and V. The animals used in these experiments are the same as those used in Chapter V where only the results for the adrenal medulla were discussed.

Animals with brain surgery were used 24 h after the operation, those with section of the spinal cord 4 days postoperatively. The operations on the brain included mesencephalic transection, diencephalic transection, hypothalamic island, and interruption of the hypothalamic link, i.e. incomplete diencephalic transection. On the day of the experiment the rats were injected intraperitoneally with 3 doses of APM, 10 mg/kg each, given at 0, 1.5 and 3 h. The rats were killed by decapitation 4 h after the initial injection.

The brain and pituitary gland were fixed in 10% formaldehyde. Serial sections of the pituitary, 4-7 μm thick, were stained with hematoxylin-eosin. Histological examination showed that no infarcts had been produced in the pituitaries of the animals that underwent surgery for the section of the hypothalamo-pituitary link. Some of the brains from each group with brain surgery were sectioned serially (100-200 μm) in a sagittal plane to check the extent of the lesion. The rest of the brain were sectioned in a sagittal plane (midline) and photographs were taken of the exposed surface.

RESULTS

Effect of spinal cord transection

The administration of APM to rats as described above and previously used in other work (2,3) leads to a considerable increase in the amount of ODC activity in the adrenal cortex (Table I, line 1). If information to and from the periphery is at all important in the regulation of this activity and of its induction, the elimination of such information by section of the spinal cord could reveal its significance for the central mechanism functioning in the APM-induced increase of ODC activity. The operations on rats to transect the spinal cord at the second and fifth thoracic vertebral levels, regions rostral to the main roots of origin of splanchnic fibers innervating the adrenal gland (2,45), were aimed at achieving this. The results of these experiments are shown in Table I, lines 2-5. First of all, it is clear that surgery of the cord, without interruption of its fibers, resulted in a mean lowering of the endogenous ODC activity in unoperated rats (lines 2 and 4, compared to line 1, for Controls). The second point in regard to the sham-operated rats is that the amount of ODC activity following treatment with APM does not attain the high levels observed in the intact rats, although it is still about four times as high as in the corresponding controls.

In contrast to these results, transection of the spinal cord led to mean endogenous ODC activities that were higher than in either sham-operated or intact rats (Table I). Moreover, the administration of APM produced greater effects in the cord-transected rats than in their sham-operated

controls. The rats with the higher transection (at T_2) appeared particularly sensitive to the interruption of all ascending and descending pathways of the spinal cord. As the induction of adrenocortical ODC activity is ultimately dependent upon the presence of the pituitary gland, these results suggest that input from the periphery to the hypophyseo-adrenocortical system, with a net inhibitory influence, is removed by interruption of ascending pathways in the spinal cord.

Rats sham-operated to control for brain operations

Twenty-four hours after brain surgery sham-operated controls showed basal levels of ODC that were comparable to those of intact controls (Table I, lines 6 and 1). Administration of APM produced a 3.5-fold increase over controls, i.e. slightly lower than that produced in intact rats (cf. lines 1 and 6) or the sham-operated animals, as recorded in lines 2 and 4 of Table I.

Mesencephalic transection

Transection of the midbrain well below the periaqueductal grey matter produced no change in the basal level of adrenocortical ODC activity. The values observed in these rats were pooled with those of the sham-operated controls to provide a mean of 49 ± 3.7 pmol CO_2/mg protein per 45 min. Administration of APM to this group of rats produced an elevation in activity similar to that in sham-operated rats (cf. lines 6 and 7).

Diencephalic transection

Transection of the diencephalon was performed in a group of rats with the aim of severing connections between mesencephalic dopaminergic centers,

viz., substantia nigra and ventral tegmentum, from the hypothalamo-pituitary unit. The fibers concerned have been described by several groups of investigators. Thus, the median eminence and the pituitary stalk contain dopaminergic fibers originating at various levels. The A_{12} dopamine system, originally described by Fuxe (18), originates in the arcuate and central periventricular nuclei. The incerto-hypothalamic system, which is essentially intradiencephalic, is composed of groups A_{11}, A_{13} and A_{14} (36). In addition, as many as half the dopaminergic fibers of the median eminence originate from perikarya in the A_8, A_9 and A_{10} cell bodies of the substantia nigra in the mesencephalon (23). Twenty-four hours after operation ODC activity of diencephalon-transected rats was significantly lower than in sham-operated controls (Table I, lines 8 and 6). The large increases produced by APM treatment of intact (line 1) and sham-operated (line 6) rats were not reproduced in animals with the diencephalon transected (line 8). Nevertheless, what increase there was could be judged statistically significant ($P < 0.05$). These results indicate that mesencephalic-diencephalic connections are necessary for maintenance of the basal levels of adrenocortical ODC activity; as well as for the normal response of this enzyme activity to the administration of APM in vivo.

Hypothalamic deafferentation

Complete hypothalamic deafferentation extending anteroposteriorly from the optic chiasma to the midmammillary bodies produced an elevation in endogenous adrenocortical ODC activity as compared to sham-operated controls (Table I, cf. lines 9 and 6). Treatment of these brain-lesioned

rats with APM increased adrenocortical ODC activity 7-fold over corresponding controls (line 9). This change was not only highly significantly different from the corresponding controls ($P < 0.001$), but also resulted in activities that were significantly different from those obtained in sham-operated rats given APM (Table I, cf. lines 9 and 6 for "APM-treated"). A parallel result obtained by Halász et al. (20) seems pertinent: they found that complete deafferentation of the medial basal hypothalamus led to the elevation of the plasma corticosteroid concentration. The significant increases of ODC activity, with or without APM treatment, to values greater than those noted in control rats suggests that this deafferentation has eliminated extrahypothalamic inhibitory influences by way of the pituitary on adrenocortical ODC activity. Moreover, the large increment in the effect of APM on adrenocortical ODC activity provides further evidence for a direct effect of the drug on receptors normally responsive to impulses along dopaminergic pathways originating in the hypothalamus and influencing the activities of the anterior pituitary gland.

Section of the hypothalamo-pituitary link

To determine whether APM has a direct effect on the pituitary gland itself, an attempt was made to separate the pituitary from the hypothalamus by the surgical procedure used to transect the diencephalon. Ideally, this lesion, extending from the rostral part of the superior colliculus ventrally to the ventromedial hypothalamic nucleus, would not only interrupt the neural connections but also damage the portal system. Endogenous adrenocortical ODC activity in rats operated in this way was slightly

higher than in sham-operated controls, but the response to APM administration was very similar to that shown by sham-operated rats (Table I, lines 10 and 6).

Histological examination of the pituitary glands of the brain-operated animals showed no infarction of the tissue. This result taken with the data on enzyme activity indicates that despite disruption of the neural connections the hypothalamic-hypophyseal portal circulation must be functional. Hence, it is not possible to deduce whether APM is acting at the level of the pituitary gland without influence from cerebral centers.

DISCUSSION

It has been previously demonstrated that the pituitary exerts a major role in the control of ODC activity of the adrenal cortex of the rat, inasmuch as hypophysectomy lowers the basal levels and prevents the induction of the enzyme normally produced by administration of APM (3). There is, nevertheless, a modulatory role of systemic information on the APM-induced increase, as indirectly demonstrated in recent unpublished work of this laboratory. These results show that adrenal demedullation, splanchnicotomy, and rhizotomy each reduces to a similar extent the response of ODC to APM administration. In the present work deafferentation of the central control mechanism by section of the spinal cord was effective in two respects: (i) in elevating the endogenous activity of the enzyme by comparison with sham-operated controls, and (ii) in increasing significantly the effect of APM in spinal animals, in particular with the section at T_2. Spinal cord section causes also an increase in the weight

and volume of the adrenal gland (15,39). Our results suggest that section of the spinal cord eliminates a net inhibitory afferent influence from the periphery on the hypophyseal system regulating ODC activity of the adrenal gland. There is morphological (21) and functional (6,13,44) evidence for a direct neural input from the adrenal glands to the hypothalamus.

On the other hand, we cannot entirely exclude the possibility that cord section eliminates an efferent inhibitory function of a supraspinal structure in relation to the adrenal cortex. There is morphological evidence for crossed efferent nerves from the basolateral hypothalamus (6,11,12,48) and from the cerebral cortex (40,52) to the adrenal cortex. Moreover, unilateral splanchnicotomy produces a slight increase in adrenocortical ODC activity of the denervated gland (unpublished results).

The next set of experiments, i.e. section of the brain at different levels, was aimed at elucidating the location of the central dopaminergic pathway(s) to the pituitary mediating the response of adrenocortical ODC activity to APM. The lesion of the transection of the mesencephalon extended from the superior colliculi ventrally into the superior cerebral peduncles. Laterally the lesion extended to the brachium colliculi inferioris at the level of the aqueduct. The structures destroyed in this lesion, in addition to those mentioned, include almost the entire mesencephalic reticular formation and the periaqueductal grey. Neither the basal level of ODC nor the APM-induced increase was significantly different from those of sham-operated controls. Direct afferents to the

lateral hypothalamus from the mesencephalic central grey matter have been described by Kuypers (28) and Szentagothai et al. (50). These authors have also described connections from the anterior midbrain reticular formation to the hypothalamus. Both areas were involved in our lesions, yet there was no alteration of adrenocortical ODC activity. From this we may conclude that the fibers travelling in the described region are not important for the APM-linked increase of this cortical function.

In another group of experiments complete transection of the diencephalon was performed to isolate the substantia nigra and ventral tegmentum from their rostral connections. The lesions extended from the rostral superior colliculus ventrally to the fossa interpeduncularis; and laterally to the fimbria hippocampi. This type of lesion significantly reduced the resting ODC levels at 24 h after the operation. The response to APM was also greatly reduced in comparison to sham-operated animals treated with the drug; but the animals nevertheless displayed a significant induction. These data indicate that for APM to have a normal effect on adrenocortical ODC activity, the ventral connections from the mesencephalon to the diencephalon must be intact. In addition, APM must also have an action at the level of the hypothalamus. In related work, Fraschini et al. (16) have shown that in midbrain-transected rats the feedback mechanism on ACTH release is less sensitive to alterations in the levels of circulating corticosteroids produced either by exogenous administration or by unilateral adrenalectomy. Their work confirmed earlier results obtained by electrolytic lesions of mesencephalic areas (14,33,35,49).

The drastic reduction in response of the adrenal cortex, with respect to ODC activity, following transection of the diencephalon may be considered along with two other findings in this work: (i) rats with section of the spinal cord maintain or even increase their response to APM by comparison with sham-operated animals; and (ii) the sham operation produced a reduction in the response to APM which is similar to the reduction caused by splanchnicotomy, adrenal demedullation, or rhizotomy (unpublished observations). On this basis we can postulate that the mesencephalon is acting as an intermediate station or relay system where peripheral inputs are processed.

The midbrain has been shown to play modulatory (16), inhibitory (8,47), excitatory (43), and stress-facilitatory (14,46) roles in the control mechanism of ACTH secretion.

The experiments aimed at isolating the hypothalamo-pituitary unit from all neural extrahypothalamic connection created "islands" that included the nucleus anterior medialis thalami and all structures ventral to this area. Anteroposteriorly, the lesions extended from the nucleus suprachiasmaticus to the posterior mammillary nucleus. In animals with complete hypothalamic deafferentation endogenous ODC levels were not only maintained but significantly increased. The APM-induced increase was also potentiated. These results demonstrate that adrenocortical ODC activity is modulated by extrahypothalamic inhibitory influences, in much the same way as has been deduced for certain endocrine functions. Thus, Egdahl (9) demonstrated that cerebral decortication of dogs elevated circulating corticosteroids

concentrations. Similar lesion and stimulation experiments involving other extrahypothalamic sites, viz. the amygdala and hippocampus, have confirmed their influence on adenohypophyseal secretion (7,10,24,31,32).

To determine whether APM is actually acting at the level of the pituitary gland to increase adrenocortical ODC activity further experiments can be suggested: (i) electrolytic lesion or section of the pituitary stalk by subtemporal or parapharyngeal approaches. These lead to functional and morphological changes of the pituitary (1,4,27) and (ii) transplantation of the pituitary to an area, distant from the hypothalamus, such as the kidney capsule.

Our results demonstrate the complexity of the neurohumoral system regulating the induction of adrenocortical ODC activity. This work has adduced the following key observations. Hypophysectomy abolishes the APM-induced increase in adrenocortical ODC activity (3). Experiments involving section of the spinal cord, together with the preparation of hypothalamic islands and mesencephalic-diencephalic transections, indicate that the primary site of response to APM must be in the diencephalon, and that its midbrain connections are required for the normal increase in adrenocortical ODC produced by the drug. Finally, it has been shown that peripheral and superior extrahypothalamic influences play a major modulatory role in this effect.

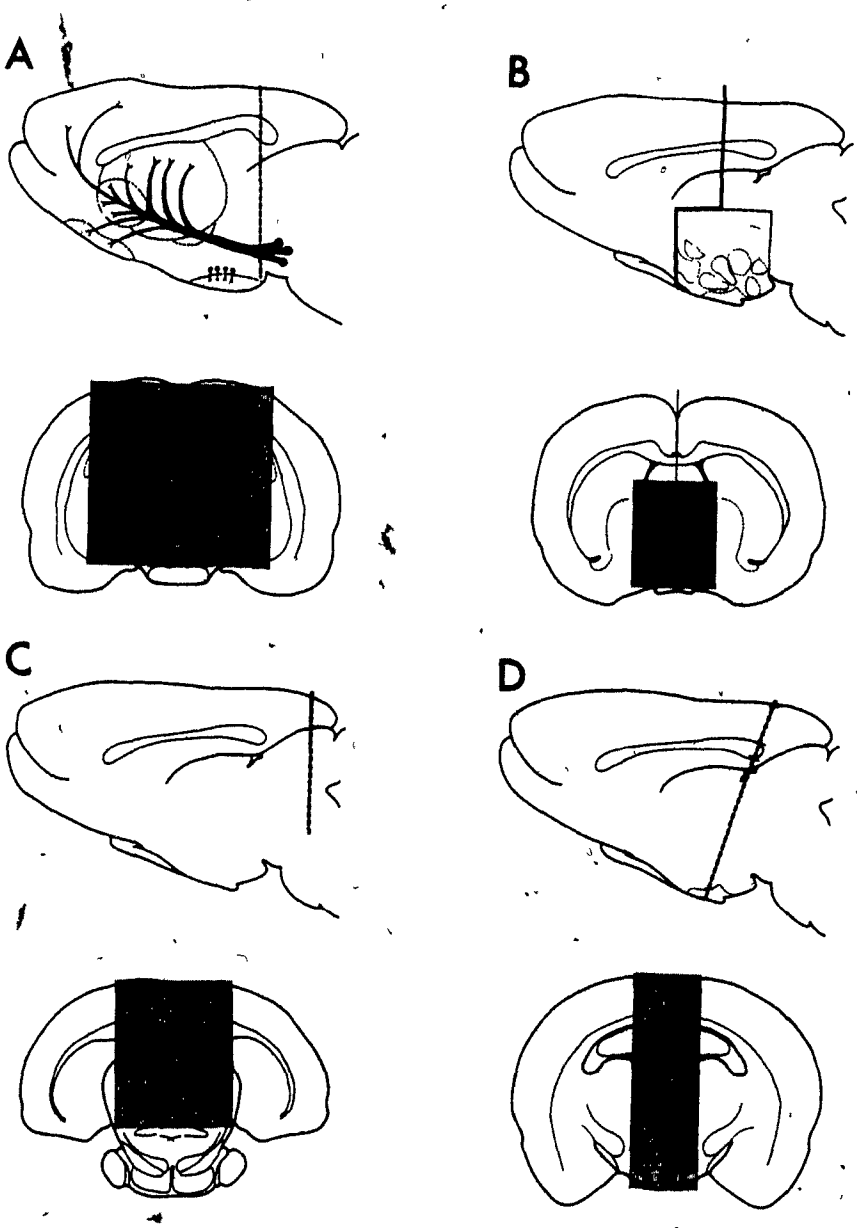

FIGURE 1

Schematic diagram of the sagittal and coronal views representing: A, diencephalic transection (main dopaminergic pathways on sagittal view); B, hypothalamic island; C, incomplete mesencephalic transection; and D, section of the hypothalamo-pituitary link.

TABLE I ODC activity in adrenocortical tissue after administration of apomorphine

Rats were injected intraperitoneally 24 h after brain section or 4 d after cord section, with APM, 10 mg/kg, at 0, 1.5 and 3 h, They were decapitated 4 h after the first injection and the adrenals removed for determinations of ODC activity, as described in the text.

[a]pmoles CO_2 produced/mg protein during incubation of tissue preparation for 45 min at 37° ± SEM.

[b]Indicates the level of significance for the difference between control and treated groups.

[c]Significantly different from the mean for sham-operated rats:

[d]$P < 0.05$; [e]$P < 0.01$; [f]$P < 0.001$.

Line	Treatment	Control		APM-treated		pb
		Mean ± SE	No. rats	Mean ± SE	No. rats	
1	Intact	67 ± 15[a]	4	269 ± 53	5	0.01
	Cord section					
2	T_5 sham	37 ± 20[a]	6	169 ± 46	6	< 0.001
3	T_5 section	124[c,d] ± 24	7	229 ± 60	7	n.s.
4	T_2 sham	20 ± 7	6	82 ± 18	4	< 0.001
5	T_2 section	96[c,f] ± 16	6	385[c,e] ± 91	5	< 0.01
	Brain section					
6	Sham	49 ± 4	6	169 ± 23	7	< 0.001
7	Mesencephalic	49 ± 4	6	140 ± 17	6	< 0.001
8	Diencephalic	27[c,d] ± 2	4	62[c,f] ± 6	6	< 0.05
9	Hypothalamic deafferentation	120[c,f] ± 12	5	848[c,f] ± 53	6	< 0.001
10	Hypothalamo-pituitary connection	69 ± 17	5	185 ± 23	7	< 0.001

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CHAPTER IX

General Conclusions

A. MECHANISM OF REGULATION OF ADRENAL ODC ACTIVITY

The administration of the dopamine-receptor agonist apomorphine, and piribedil, to rats leads to an increase in the activity of ODC of the adrenal medulla and cortex. These changes in enzyme activity are mainly due to protein synthesis de novo as deduced from the following observations:

(i) Kinetic studies of dialyzed enzyme preparations from control and experimental rats showed similar K_m values; this was for both the adrenal medulla and cortex.

(ii) Mixing experiments, in which enzyme preparations of controls and APM-treated rats are incubated together, were always additive. This indicates that the increase in ODC activity is not caused by the presence of an activator or the loss of an inhibitor.

(iii) Pretreatment of the rats with the protein synthesis inhibitor cycloheximide prevented the large increase in ODC activity normally seen after APM administration in both adrenal structures.

Kinetic studies revealed the presence of two distinct K_m values for the cofactor pyridoxal 5'-phosphate. This indicates that ODC may be present in two different forms in the adrenal glands. The significance and interrelationship between these two forms of ODC for the regulation of the enzyme activity is at present not well understood and deserves further study.

B. CENTRAL DOPAMINERGIC PATHWAYS INVOLVED IN THE INDUCTION OF ADRENOMEDULLARY ODC ACTIVITY

Adrenomedullary ODC activity is predominantly regulated by a central

system, originating mainly in the diencephalon-telencephalon and including a facilitatory dopaminergic component. Nerve impulses evoked by the action of the dopaminergic agonists travel in a pathway lateral to the hypothalamus, in the ventral part of the midbrain, down to the spinal cord, and finally affect the adrenal medulla by way of the splanchnic nerve. This is supported by the following observations:

(i) Pretreatment of the rats with the dopamine receptor-antagonist haloperidol (HLP) causes a partial blockade of the APM-induced effect at 4 h in intact animals and at 6 h in hypophysectomized.

(ii) Denervation of the adrenal gland prevents the increases in ODC activity produced by either APM or PBD at 4 h, and the early increases produced by a large dose of HLP.

(iii) Section of ventral spinal roots reduces the inductive effect of APM administration, the reduction depending upon the number of roots interrupted and their location in the thoracic region from T_4 to T_{12}.

(iv) Section of the spinal cord above the main roots of origin of the splanchnic fibers almost prevents the increase produced by APM.

(v) Transection of the diencephalon-mesencephalon reduces endogenous ODC activity and abolishes the induction by APM.

(vi) Section of the dorsal part of the midbrain does not change either endogenous or APM-induced ODC activity.

(vii) Formation of a hypothalamic island not only maintains but even elevates the response to APM. This indicates that the pathways are travelling more lateral to the lesion.

C. CENTRAL DOPAMINERGIC PATHWAYS INVOLVED IN THE INDUCTION OF ADRENOCORTICAL ODC ACTIVITY

Induction of adrenocortical ODC activity involves a central dopaminergic pathway originating mainly in the hypothalamus. Its action is mediated by the release of ACTH from the pituitary, and is modulated by both peripheral and central extrahypothalamic influences. Support for this hypothesis arises from several sets of observations:

(i) Pretreatment of rats with HLP causes a partial blockade of the APM-induced increase in adrenocortical ODC at 4 h.

(ii) Intraventricular injection of the neurotoxin 6-hydroxydopamine to destroy dopaminergic nerve terminals causes a significant reduction in the APM effect.

(iii) Hypophysectomy completely prevents the rise in ODC activity observed in intact rats after APM administration and reduces endogenous ODC. Administration of ACTH to hypophysectomized animals not only restores the basal activity but induces the enzyme maximally at 12 h.

(iv) Surgical manipulation of the animals such as unilateral splanchnicotomy or sham-operation at the level of the spinal cord (without damage to neural elements) attenuates the response to APM. By contrast, section of the spinal cord increases endogenous and APM-induced ODC activity.

(v) Bilateral adrenal demedullation reduces the ODC response to APM and suppresses the time-dependent increases observed in intact rats.

(vi) Formation of a hypothalamic island produces a significant increase in endogenous ODC activity and potentiates the effect of APM.

D. DOPAMINERGIC-SEROTONERGIC INTERACTION

The functional interaction of serotonergic with dopaminergic systems provides net opposite effects on adrenomedullary and adrenocortical ODC activities: inhibitory for the medulla and facilitatory for the cortex. This is supported by the following experiments:

(i) Systemic administration of p-chlorophenylalanine (PCPA), intraventricular injection of 5,6-dihydroxytryptamine (DHT) and selective electrolytic lesions of the medial raphe nucleus, all of which deplete 5HT by different mechanisms, cause a potentiation of the APM increase in adrenomedullary ODC activity.

(ii) On the other hand, both drugs and the electrolytic lesion of the medial raphe decrease the adrenocortical ODC response to APM.

(iii) The medial raphe nucleus is involved in both systems.

Finally, the increase in adrenomedullary ODC activity caused by the administration of dopaminergic drugs involves at least three types of neurons: dopaminergic, serotonergic and the cholinergic sympathoadrenal preganglionic neurons. Our results show that changes in ODC activity in the adrenal medulla reflect predominantly alterations in the activity of the sympathoadrenal system, whereas the changes in the cortical enzyme reflect modifications of the hypothalamo-pituitary system. Furthermore, the model explored in this thesis may serve as a unique tool to study the regulatory relationship between the two adrenal structures in their response to stress, and similarly, to trace specific neural pathways.

CHAPTER X

Original Contributions to Knowledge

1. Administration of apomorphine (APM) or piribedil (PBD), two dopamine receptor agonists, to rats causes a time-dependent increase in the activity of ODC of the adrenal medulla and of the cortex. The effect of APM on both structures is dose-related and appears to involve induction of protein synthesis.

2. Kinetic studies suggest that the adrenal gland contains two different forms of ODC with different constants of affinity for the cofactor but similar constants of affinity for the substrate.

3. Pretreatment of rats with a small dose of the dopamine-receptor antagonist, haloperidol (HLP), causes blockade of the increase in ODC produced by the agonists APM or PBD for the first four hours after their administration. This makes evident the involvement of a dopaminergic system or systems in the regulation of adrenal ODC activity. This dopaminergic function is excitatory for both adrenal medulla and cortex, with respect to ODC activity.

4. A larger dose of HLP than is required to block the action of APM or PBD induces ODC activity in both the adrenal medulla and cortex in a time-dependent manner. The medullary increase observed at 2.5 h is prevented by splanchnicotomy. The results indicate that HLP-induced increase in adrenomedullary ODC activity is caused by a reflex increase in preganglionic nerve activity.

5. Hypophysectomy reduces endogenous ODC levels in the cortex, but not in the medulla. The effect of APM on adrenocortical ODC activity is abolished in hypophysectomized rats. Treatment of hypophysectomized rats with ACTH not only restores but induces the adrenocortical enzyme in a time-dependent manner. This demonstrates that adrenocortical ODC is regulated by the pituitary gland, but that the medullary enzyme is not. The action of APM on cortical ODC is ultimately mediated by the pituitary, most probably through the release of ACTH.

6. Unilateral section of one to four ventral spinal roots from T_4 to T_{12} partially prevents the APM-induced increase in adrenomedullary ODC activity. Dorsal root section at T_7-T_{10} leads to a small reduction, while section at T_2-T_4 has no effect at all. Thus, selective surgical interruption of spinal roots indicates that the bulk of splanchnic fibers mediating the transynaptic induction of adrenomedullary ODC course in the ventral roots between $T_{\wedge\wedge}$ and T_{10}. Dorsal rhizotomy causes similar changes; these manifest the existence of a segmental modulatory function (or reflex mechanism) of the afferent input to the spinal cord in regard to sympatho-adrenal preganglionic neurons involved in innervation of the chromaffin cells. This novel functional method of tracing neuroanatomical pathways should be applicable to tracing the origin of fibers regulating other activities of the adrenal medulla.

7. The APM-induced increase in adrenomedullary enzyme activity is reduced very significantly (but not completely) by unilateral splanchnicotomy, section of the spinal cord at T_5 and T_2 or complete transection of the diencephalon. Transection of the dorsal mesencephalon does not affect the induction. However, incomplete transection of the diencephalon and formation of a hypothalamic "island" potentiate the effect produced by APM. These results indicate that the central dopaminergic pathway involved in the regulation of adrenomedullary ODC includes important nuclei in the diencephalon-telencephalon. A second level of control appears to be the thoracic cord because its section does not completely prevent the APM-induced increase in ODC activity.

8. Depletion of cerebral serotonin (5HT) by administering rats the tryptophan hydroxylase inhibitor p-chlorophenylalanine (PCPA), or destruction of 5HT nerve terminals by injection of 5,6-dihydroxytryptamine (DHT) into the cerebroventricular system of the rat, produces no change in ODC activity. However, both treatments potentiate the induction of adrenomedullary ODC produced by APM. The results indicate that a central serotonergic system exerts an inhibitory role over the APM induction of ODC.

9. Electrolytic lesions of the medial raphe nucleus, but not of the dorsal raphe, potentiate the APM-induced increase in adrenomedullary ODC. Thus, the medial raphe nucleus is involved in this functional dopaminergic-serotonergic interaction.

10. Unilateral splanchnicotomy, rhizotomy, or bilateral demedullation each attentuate the response of adrenocortical ODC to APM. Intraventricular injection of 6-hydroxydopamine, which destroys dopaminergic nerve terminals, reduces the effect of this drug. On the other hand, section of the spinal cord or formation of a hypothalamic "island" potentiate that effect. The results suggest that the central dopaminergic system mediating the APM-induced increase in adrenocortical ODC activity is located mainly within the hypothalamus. However, peripheral and extrahypothalamic central nervous influences play a modulatory role in this effect.

11. Administration of PCPA, DHT or selective electrolytic lesion of the medial raphe nucleus reduces the APM-induced increase in adrenocortical ODC activity. The results suggest that serotonergic structures originating in the medial raphe nucleus play a facilitatory role in the response of adrenocortical ODC to APM.