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IN VITRO PROGESTERONE METABOLISM BY SELECTED BRAIN AREAS, PITUITARY GLAND AND TESTES OF THE ADULT MALE CAT*

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SUMMARY

This study was conducted to define the pattern of *in vitro* metabolism of [^{3}H]-progesterone by the adult cat reproductive system involving the following structures: selected brain areas implicated in reproductive physiology, anterior pituitary, and the testes. Tissues were incubated with radiolabeled progesterone, and the formed metabolites were separated, isolated, and purified by sequential paper chromatography, and further identified by derivative formation and isotopic dilution and recrystallization. All tissues used in this experiment converted progesterone to androgens, indicating the presence of 17 α-hydroxylase, 17, 20-desmolase and 17 β-hydroxysteroid dehydrogenase; some tissues also demonstrated a capacity to produce estrogens, showing the presence of the aromatizing enzyme system; yet, no 5α-reduced androgens were detected in any of the incubations. These transformations in neuroendocrine tissues may provide further evidence concerning the possible role of androgens and estrogens in central reproductive physiology and sexual behavior.

INTRODUCTION

The brain may be considered a target tissue for the action of steroid hormones which affect pituitary function and behavior[1, 2].

The mechanisms of hormone action and their effects on different areas of the brain are not well understood. To learn more about those effects, special emphasis should be placed on determining the brain tissue levels of enzymes involved in the biosynthesis and bioconversion of steroid hormones.

Although many investigators have demonstrated the conversion of testosterone and androstenedione to different metabolites by the pituitary gland and by selected areas of the brain of male and female rats[3–9], and of male and female rats, mice, rabbits, rhesus monkeys, and humans[10, 11], studies with progesterone, used as precursor in incubations, were mostly performed with female brains[12–14], and rarely with male brains[16]. A progesterone derivative was also used as a precursor in one experiment with female brains[15].

A considerable body of evidence[10, 11, 17] has accumulated to suggest that many central reproductive functions of androstenedione and testosterone may require their conversion to estrogens. The above-mentioned experiments deal primarily with the bioconversion of androgens by the brain of male and female animal species, or the metabolism of progesterone by the female brain. However, the present study was designed to investigate the enzymatic capacity and activity of different areas of the male brain—especially those which may be implicated in sexual behavior and central reproductive physiology—to convert [^{3}H]-progesterone to androgens and estrogens. The male cat brain was chosen because (1) pertinent research in the central reproductive mechanisms using the cat has not been adequately developed, and (2) the large cat brain also allows us to analyze the specific brain areas closely linked to sexual behavior and central reproductive physiology.

MATERIALS AND METHODS

Three adult male cats (2.4–4.0 Kg) were utilized in the present investigation. Some special techniques were developed to permit neuroanatomic dissection of the cats' brains[18]. As part of this procedure, cats were sacrificed by cutting the spinal cord; the pituitary gland, the whole brain and the testes were then carefully removed. The brain was sectioned with a

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The following trivial names and abbreviations are used in this paper:

Androstenedione (A): 4-androsten-3,17-dione;

17β-Estradiol (E_2): 1,3,5(10)-estratriene-3,17β-diol;

Estrone (E_1): 3-hydroxy-1,3,5(10)-estratriene-17-one;

17α-Hydroxyprogesterone (17α-OH-P): 17α-hydroxy-4-pregnene-3,20-dione;

Progesterone (P): 4-pregnene-3,20-dione;

Testosterone (T): 17β-hydroxy-4-androsten-3-one;

NADP, Na: nicotinamide adenine dinucleotide phosphate, sodium salt;

G-6-P, Na_2: glucose-6-phosphate, disodium salt.

scalpel and various spatulas were utilized to remove specific, easily identifiable areas. This study examines the conversion of [^{3}H]-progesterone to androgens and estrogens by the pituitary gland, selected areas of the brain (nucleus accumbens, caudate nucleus, hippocampus, hypothalamus, frontal and occipital cortex) and the testes.

A. *Chemicals.* The radiolabeled steroids were purchased from New England Nuclear Corporation (Boston, MA, U.S.A.) and purified by paper and thin-layer chromatography. The [^{3}H]-progesterone, supplied at a specific activity of 20 μCi/mmol was diluted with unlabeled steroid to yield 2.61 μCi/mmol; 22×10^6 d.p.m., 3.92 nmol were used per incubation flask. The [^{14}C]-labeled androstenedione, estrone, estradiol, 17α-hydroxyprogesterone, progesterone, and testosterone, supplied at a specific activity of 50 μCi/μmol each, were diluted with absolute ethanol in order to yield approximately 40,000 d.p.m./ml solution.

Analytical grade solvents were distilled before use.

Non-radioactive steroids, obtained from Steraloids, Incorporated (Pawling, NY, U.S.A.) were purified on silica gel column and recrystallized to their respective melting points. Whatman No. 1 chromatographic paper, and silica gel precoated aluminium sheets were used for paper chromatography and thin-layer chromatography respectively.

B. *Incubation techniques.* Selected brain areas (nucleus accumbens, caudate nucleus, hippocampus, hypothalamus, frontal and occipital cortex), as well as pituitary and testes were removed from the cats and placed into chilled Petri dishes. Fifty to 100 mg of tissue were placed in a flask containing 1.5 ml of incubation medium and [^{3}H]-progesterone (approx. 22×10^6 d.p.m., 3.92 nmol); the tissue was gently teased with scissors and incubated in a Dubnoff metabolic shaker for 3 h at 37°C under air. The incubation was terminated by the addition of 10 ml ethyl acetate and, at the same time, appropriate ^{14}C-tracers (approximately 40,000 d.p.m. of each) were added to the mixture to correct for losses in subsequent manipulations. The material was frozen until further processing.

Composition of incubation medium (for total volume of 1.5 ml): 1 ml Hanks' solution, 25 μl ethanol, 175 μl NADP, Na solution (3.257 μmol/ml), 175 μl glucose-6-phosphate, Na_2 solution (36.17 μmol/ml), 125 μl 1.3% $NaHCO_3$.

C. *Extraction procedure.* The incubation mixture was thawed and extracted with ethyl acetate, partitioned with hexane–70% methanol, and finally extracted with chloroform. The appropriate radioinert carriers (50–100 μg each) were added to the dry residue obtained from the chloroform extraction.

D. *Paper chromatography and identification of metabolites.* The dry residue obtained from the chloroform extraction was paper chromatographed in Bush A system, heptane–methanol–water (100:80:20, by vol) against a number of appropriate standards. The 4-ene-3-ketosteroids (carriers and standards) were located on the paper chromatography strips by means

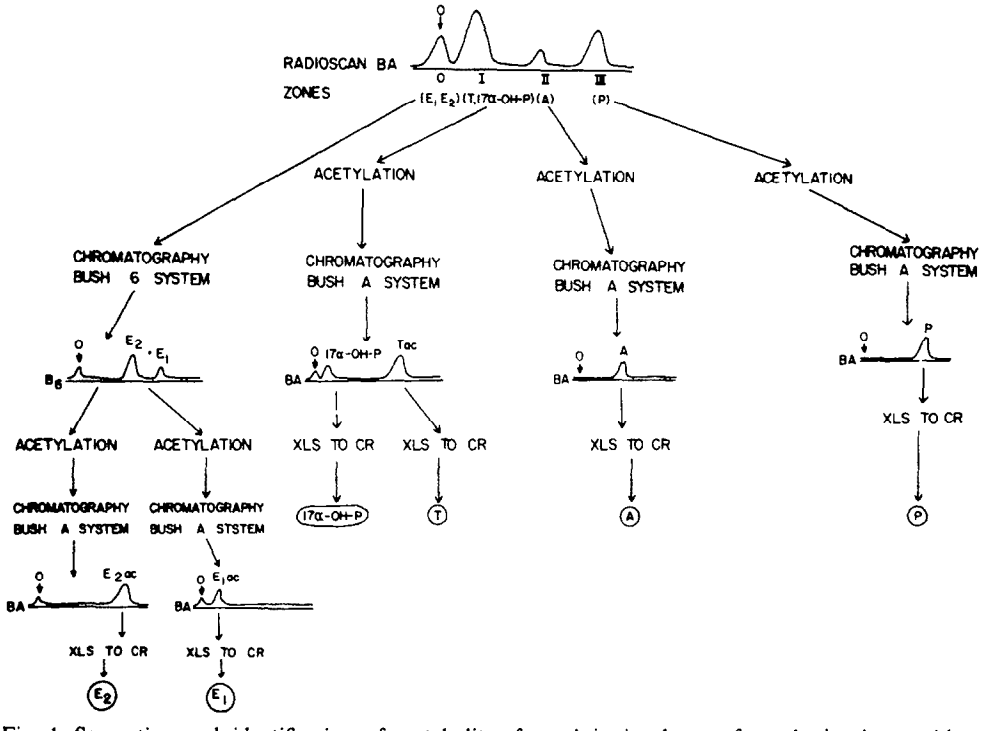

Fig. 1. Separation and identification of metabolites formed in incubates of cat brain tissue with [^{3}H]-progesterone. The diagram illustrates the distribution of radioactivity in the first chromatogram (Bush A) and subsequent steps involved in purification and identification of the metabolites. Abbreviations: XLS = crystallization, CR = constant ^{3}H/^{14}C ratio.

of a short-wave ultraviolet lamp. On the standard chromatography strips, the androgens were located by spraying with Zimmermann's reagent. The chromatograms from the chloroform extracts were scanned in a Nuclear Chicago, model Actigraph III, radiochromatogram scanner. Identification of the different steroids was accomplished by formation of derivatives and by the technique of isotopic dilution and recrystallization to constant $^3H/^{14}C$ ratio after addition of 50–100 mg of appropriate carrier steroid. Crystallization was carried out in several solvent mixtures. The labeled compound was considered radio-chemically pure and identical with the carrier steroid if the $^3H/^{14}C$ ratio in 2 or 3 consecutive samples of the crystals did not deviate by more than 5–8% from the mean value.

Because all tissues used in this experiment showed similar patterns in the initial chromatography in Bush A system, the subsequent steps toward the purification and identification of the different metabolites were practically the same. Figure 1 diagrams the processing of the different radioactive zones after initial chromatography in Bush A system.

E. *Acetylation.* The dry material was dissolved in 0.1 ml pyridine, to which 0.1 ml acetic anhydride was added, and the reaction mixture was left overnight at room temperature; 0.2 ml methanol was added to stop the reaction and the solution was evaporated to dryness. The dry residue was then chromatographed in the appropriate system.

F. *Measurement of radioactivity.* Radioactivity of ^3H and ^{14}C standards and of samples of eluted steroids after chromatography, and of crystals and mother liquors after recrystallization, was measured by counting in a Intertechnique, model SL 30, liquid scintillation spectrometer, set for simultaneous counts of ^3H and ^{14}C. The samples were prepared by dissolving an aliquot of the dried eluate, or the crystals, or the dried mother liquor, in 10 ml scintillation solution (0.4% w/v of 2.5-*bis*-{2′ (5′-tertbutylbenzoxazolyl)}-thiophene in toluene). The radioactivity of the substrates and the tracers was measured in the same instrument by setting it for single counts. Quenching was usually absent in the samples counted by the above-mentioned procedure.

G. *Computations.* In cases of metabolites where ^{14}C-labeled tracers were utilized, the percent conversion of the substrate ([^3H]-progesterone) to the different metabolites was expressed in terms of radioactivity or weight. This was calculated after isotopic dilution and recrystallization to constant $^3H/^{14}C$ ratio, using the following formulas:

$$\% \text{ conversion} = \frac{\text{ratio } ^3H/^{14}C \text{ in last crystals} \times \text{d.p.m. } ^{14}C\text{-tracers}}{\text{d.p.m. } ^3H\text{-substrate}}$$

$$\% \text{ conversion} \times n\text{mol substrate} \times 10 = p\text{mol metabolite in incubation}$$

$$\frac{p\text{mol metabolite in incubation} \times 1000}{\text{weight of tissue (mg) in incubation}} = p\text{mol metabolite/g tissue}$$

RESULTS

All tissues used in the present experiment were found to have the capacity to convert progesterone to androgens; this would indicate the presence of 17α-hydroxylase, 17,20-desmolase and 17β-hydroxysteroid dehydrogenase in the tissues. Most of the tissues also converted progesterone to estrogens, thus uncovering the presence of an active aromatizing enzyme system. The final results are expressed in two ways, (1) percentage of conversion of progesterone to the different metabolites and (2) pmol of metabolite per gram of tissue used in the incubation process. The final values represent the mean for the three cats used in the experiment.

Chromatography performed on all the samples indicated four radioactive peaks: zone 0, or polar zone, at the origin, which contains all the polar steroids; zone I, corresponding in chromatographic mobility to testosterone and 17α-hydroxyprogesterone; zone II, corresponding in mobility to androstenedione; and zone III, corresponding in mobility to progesterone.

As expected, the testicular tissue was the one which most actively metabolized progesterone (more than 95% of progesterone metabolized), whereas, the pituitary gland and selected areas of the brain metabolized progesterone at a rate between 10 and 15%. Table 1 presents the recovered radioactivity in the different zones after the first chromatography in Bush A system.

The zone 0, or polar fraction, re-chromatographed in B6 system, toluene–methanol–water (100:75:25, by vol.) resulted in two radioactive zones, the more polar, corresponding in chromatographic mobility to estradiol, and the less polar, to estrone. These two zones were acetylated and chromatographed again in Bush A system. Those areas, corresponding in chromatographic mobility to estrone acetate and estradiol diacetate, were eluted and recrystallized to constant $^3H/^{14}C$ ratio. Although it was not possible to detect estrone in any of the samples (probably because of the very small amounts present), yet, estradiol was detected and identified in most of these preparations.

Zone I, corresponding in chromatographic mobility to testosterone and 17α-hydroxyprogesterone, was eluted, acetylated, and re-chromatographed in Bush A system. Two radioactive peaks were detected, one more polar, corresponding in chromatographic mobility to 17α-hydroxyprogesterone, and the other, less polar, with a chromatographic mobility similar to testosterone acetate. Both compounds were further re-

Table 1. Radioactivity recovered in the different zones after the first chromatography in Bush A system

Tissue	Percentage of radioactivity recovered after the first chromatography in Bush A system			
	Zone 0	Zone I	Zone II	Zone III
Pituitary	12	2.6	1.0	84
Caudate nucleus	16	4.2	1.1	78
Nucleus accumbens	16	5.4	1.8	77
Hippocampus	11	3.6	1.0	84
Hypothalamus	12	2.3	0.8	85
Cortex (frontal)	14	5.0	1.3	80
Cortex (occipital)	19	4.2	1.5	76
Testes	74	12	9.0	5.2

Zone 0 = polar steroids. Zone I = testosterone and 17α-hydroxyprogesterone. Zone II = androstenedione. Zone III = unconverted progesterone.

crystallized to constant $^3H/^{14}C$ ratio, and identified as 17α-hydroxyprogesterone and testosterone acetate.

Zone II, corresponding in chromatographic mobility to androstenedione, was eluted, acetylated, and re-chromatographed in Bush A system, where only one peak was detected. This peak, after elution and recrystallization to constant $^3H/^{14}C$ ratio, was found to be androstenedione.

Zone III, with chromatographic mobility similar to that of progesterone, underwent the same process as zone II, and again only one peak, identified as progesterone (unconverted substrate), was present after acetylation. In all the incubations performed in this study and under the present experimental conditions, no 5α-reduced androgens were detected, suggesting the absence or inactivity of 5α-reductase in the tissues used.

The various areas of the brain, the pituitary and testes showed different enzymatic activity in converting progesterone to the above-mentioned metabolites; this activity, expressed in percentage of conversion as radioactivity or as weight and as pmol of metabolite per gram of tissue, is presented in Table 2 and in Fig. 2.

A baseline pattern of the enzymatic capacity of selected areas of the brain in converting progesterone to different metabolites was established. Based on the available data in the literature and our own results, a tentative metabolic pathway from progesterone to androgens and estrogens is presented in Fig. 3.

DISCUSSION

Results of the present study clearly show that all the tissues converted progesterone to androgens, but with different levels of activity, thus indicating the presence of 17α-hydroxylase, 17–20 lyase, and 17β-hydroxysteroid dehydrogenase. Most of the tissues also demonstrated a capacity to convert androgens to estrogens. This would indicate the presence of the aromatizing enzyme system. The conversion of androgens to estrogens is of extreme importance for the reproductive functions of androstenedione and testosterone[10, 11]. No 5α-reduced androgens, however, were detected in any of these incubated preparations. This negative finding suggests two possible conclusions: It may be that 5α-reductase was absent in the tissues, or that the enzymatic activity was too low to be measured under our experimental conditions.

A great deal of information is available concerning the action of steroid hormones upon the pituitary gland and the brain. For example, the existence of feedback mechanisms between the pituitary gland and

Table 2. Conversion of progesterone to testosterone, 17α-hydroxyprogesterone, androstenedione and estradiol by the pituitary, selected areas of the brain and testes

Tissue	Conversion of progesterone to							
	Testosterone		17α-OH-P		Androstenedione		Estradiol	
	%	pmol/g tissue	%	pmol/g tissue	%	pmol/g tissue	%	pmol/g tissue
Pituitary	0.13	67	0.51	250	0.45	222	0.07	34
Caudate nucleus	0.13	54	0.26	110	0.42	173	0.08	34
Nucleus accumbens	0.14	105	0.31	240	0.48	374	ND	ND
Hippocampus	0.13	78	0.24	138	0.29	168	0.08	47
Hypothalamus	0.07	87	0.16	212	0.31	394	0.08	102
Cortex (frontal)	0.08	36	1.86	785	0.46	193	0.08	35
Cortex (occipital)	0.08	34	0.54	218	0.36	146	0.08	31
Testes	2.30	624	0.57	150	4.96	1,142	0.07	29

The results represent the mean for the three cats used in the experiment. ND = not detectable

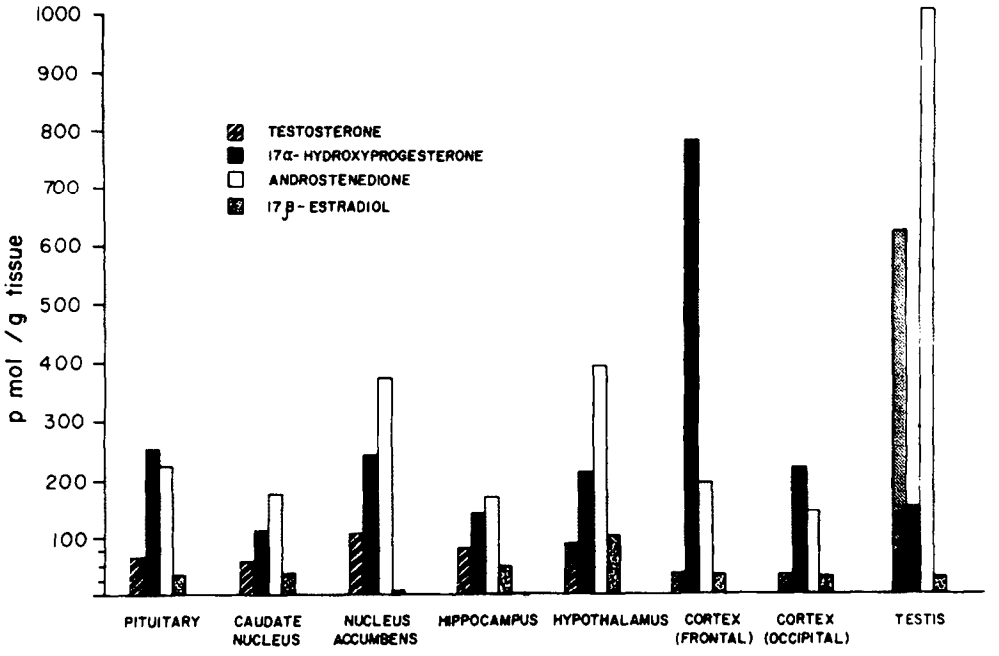

Fig. 2. [^{3}H]-Progesterone conversion to different metabolites by selected areas of the brain, pituitary gland and testes, expressed as pmol/g tissue.

the gonads, and more recently, between the hypothalamus and the gonads, have been firmly established. It is clear that steroid hormones have profound effects on the brain: gonadal hormones control sex differentiation, trigger puberty, and regulate adult sexual behavior; adrenal steroids influence mood, sensory perception, instinctive and adaptive behavior[19]. Some hormones act directly, without being metabolized and others need to be converted to certain metabolites in order to exert their effects on the brain[2].

Several investigators[3–11] have studied the conversion of testosterone and androstenedione by the anterior pituitary and various areas of the brain. Our experiments went a few steps further back in the steroid metabolic pathway as we studied the enzymatic capacity of the pituitary and selected brain areas of the adult male cat to metabolize progesterone *in vitro*, and, in turn, this activity was compared with the metabolic capacity of the testes. We demonstrated further that the male cat brain has comparable enzymatic capacity to metabolize progesterone, and that this capacity is qualitatively similar to that of the testes, differing only quantitatively.

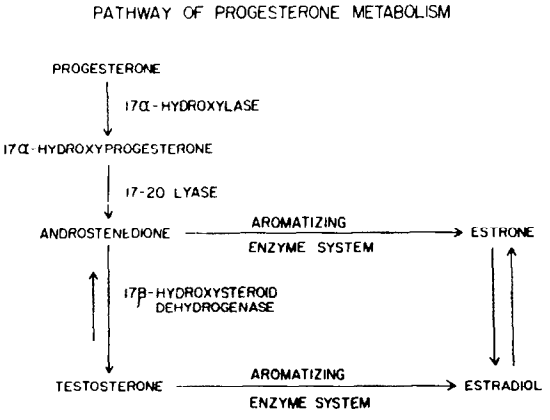

Fig. 3. Tentative metabolic pathway from progesterone to androgens and estrogens.

Previous experiments in our laboratories[20–22] dealt with the conversion of progesterone by the rat testicular tissue at different stages of development. We have found that progesterone was avidly metabolized regardless of the age of the animal, but that the pattern of metabolism varied markedly, depending on the stage of testicular differentiation. Striking quantitative changes in the conversion of progesterone to androgens, especially around the time of the onset of puberty, were observed; this is the period (between 20 and 40 days of age in the rat) where 5α-reductase is most active, and large amounts of 5α-reduced androgens are produced[20, 21]. In the present study our tissues were from adult cats, and we speculate that this may be the reason for not finding 5α-reductase activity. On the other hand, we were able to demonstrate that the *in vitro* conversion of [^{3}H]-progesterone by the adult cat testes is, qualitatively and quantitatively, analogous to that of the adult rat testes[21].

Having some preliminary evidence (unpublished data from our laboratories) that similar age-dependent changes occur when key areas of the brain and anterior pituitary are incubated with progesterone, our next step will be to study serially the enzymatic activity of the same areas of the brain included in the present study, but as a function of age. We hope that these longitudinal experiments may provide a better understanding of the relationship between the

brain and the hormones produced by the gonads, the onset of puberty, and the endocrine mechanisms in the brain directly involved in central reproductive physiology and sexual behavior.

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