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Effect of Acute Polybrominated Biphenyl Treatment or Orchidectomy on Hypothalamic and Hepatic Androgen Metabolism

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EFFECT OF ACUTE POLYBROMINATED BIPHENYL TREATMENT OR ORCHIDECTOMY ON HYPOTHALAMIC AND HEPATIC ANDROGEN METABOLISM

By

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ABSTRACT

EFFECT OF POLYBROMINATED BIPHENYL
TREATMENT OR ORCHIDECTOMY ON HYPOTHALAMIC
AND HEPATIC ANDROGEN METABOLISM

By

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Reproductive effects seen in PBB exposed animals may be related to increased steroid metabolism. To determine whether PBB effects on reproduction also involved the hypothalamus, hypothalamic androgen aromatization (HA) was measured. Rats were treated with a single dose of Firemaster BP-6 (150 mg/kg i.p.), assigned to a control group, or orchidectomized at 21 days of age. HA activity was measured in adult rats by incubation of hypothalamic homogenate with ^{3}H-androstenedione, and the ^{3}H-estrone produced was measured by liquid scintillation counting following purification by two successive HPLC steps. In vitro incubations of testosterone with liver microsomes confirmed PBB induction of steroid metabolism. HA activity in control males was approximately 3 times greater than the activity in control females. Prepubertal castration caused a decrease in HA activity in male rats but not to levels seen in female rats. PBB treatment resulted in a trend toward decreased aromatization in male and female rats.

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INTRODUCTION

Polybrominated Biphenyls as Reproductive Toxicants

Michigan's polybrominated biphenyl (PBB) contamination problem began during 1973 with the accidental incorporation of a commercial PBB mixture into cattle feed. Accidental shipment of "Firemaster BP-6" from the St. Louis, Michigan plant of Michigan Chemical Corporation resulted in the incorporation of polybrominated biphenyls into at least three lots of cattle feed produced by the Michigan Farm Bureau. Estimates of PBB concentration in the contaminated feed have ranged as high as 13,500 ppm (Kay, 1977). Feed consumption by cattle, and the subsequent distribution of contaminated meat and dairy products throughout the state have resulted in the exposure of most of Michigan's population to PBB. Toxicological studies have determined that the number of Michigan residents with detectable PBB levels ranges from as low as 43% in the upper peninsula to as high as 96% in the lower peninsula (Eyster, 1976). Following the discovery of the PBB contamination in 1973, scientific and anecdotal reports of adverse human and animal health

effects of PBBs began to occur.

The study of the PBB contamination has been complicated by the fact that PBBs, like the chemically and toxicologically related PCBs (polychlorinated biphenyls), are not produced or distributed as single compounds, but rather as complex mixtures of polyhalogenated biphenyl compounds. In addition, other non-biphenyl constituents such as polybromonapthalenes or polybromodibenzofurans may be present (Dent, 1978), or may be formed during cooking or processing of PBB contaminated foods (O'Keefe, 1978). Firemaster BP-6 is a mixture of at least 18 PBB congeners of varying degrees of bromination (DiCarlo et al, 1978). The major congeners present in Firemaster BP-6 are 2,4,5,2',4',5'-hexabromobiphenyl (approximately 60%) and 2,3,4,5,2',4',5'heptabromobiphenyl (11 - 27%) (Dent, 1978). Tetra-, penta-, octa-, and higher bromobiphenyls are also present (Harris et al, 1978).

The effects of polybromobiphenyls on human and animal health have been the subject of some controversy. Unconfirmed reports of non-specific toxicosis and malaise in both exposed humans and cattle have been reported in the popular press, and in the scientific literature. High dose feeding of PBBs to cattle have resulted in anorexia, diarrhea, salivation and lacrimation, fetal death, "general depression", skin, hoof, and kidney lesions, and elevated SGOT, SGPT, and BUN (Durst et al, 1978; Roble et al, 1978). Anecdotal reports of PBB toxicosis in humans

have listed symptomatology including numbness, nausea, weight loss, anorexia, hepatitis, fainting, headache, fatigue, irritability, depression, acne, and a variety of other symptoms (DiCarlo et al, 1978). Michigan Department of Public Health studies found "...no consistent pattern of illness or symptoms..." (DiCarlo et al, 1978), but subsequent human studies have identified at least a few health effects probably linked to PBB ingestion. Toxicological studies of PBB exposed Michigan residents have revealed immuno-deficiencies in both humoral and cell-mediated responses (Bekesi, 1979), and increased incidence of halogenacne among exposed individuals (DiCarlo et al, 1978). Other studies have shown a general impairment of good health among exposed children (Barr, 1978), and a variety of clinical abnormalities in liver function (DiCarlo et al, 1978) in exposed adults. The clinical picture is a complicated one, however, and some investigators still maintain that there exists "... no relationship between PBB levels and physical or laboratory findings...[exposed individuals have]...few objective findings and reactive depression may be responsible for the high prevalence of constitutional symptoms." (Stross, 1981).

PBBs are known potent inducers of hepatic and extrahepatic mixed-function oxidase (MFO) enzyme metabolism (McCormack et al, 1978; Dent, 1978; Kluwe et al, 1978; Goldstein et al, 1979; Dent et al, 1978; Robertson et al, 1981; Dent et al, 1976; Babish and Stowesand, 1977). The

PBB mixture, Firemaster BP-6 is an established "mixed" inducer of hepatic mixed function oxidases, with an effect similar to the simultaneous administration of both phenobarbital (PB), a P-450 inducer, and 3-methylcholanthrene (3-MC), a P-448 inducer. Treatment with PCB mixtures results in a pattern of MFO induction virtually identical to that seen with combined phenobarbital and 3-MC treatment (Ryan et al, 1977). Microsomal proteins produced by PBB treatment, however, have been shown to differ slightly from those produced by concurrent phenobarbital and 3-MC treatment by SDS-PAGE electrophoresis (Dent, 1978).

Treatment of animals with proven inducers of MFOs such as phenobarbital or PCBs has led to increased microsomal metabolism of steroid hormones (Nowicki and Normal, 1972; Orberg and Kihlstrom, 1973; Orberg and Lundberg, 1974; Derr, 1978; Sanders et al, 1974). This increased steroid metabolism may be responsible in part for the alterations in reproductive functions seen in PCB treated animals. In rodent systems, PCBs have produced uterine atrophy, reductions in plasma progesterone concentration, lengthened estrous cycles, and additional reproductive effects (Jonsson et al, 1976; Kimbrough et al, 1978;). Recent reports in the literature have shown that administration of phenobarbital during development results in permanent reproductive deficits (Gupta et al,1980; Gupta et al, 1982) including permanent reductions in circulating testosterone levels.

The MFO inducing properties of PB and PCBs are shared by PBBs. Several endocrine-related alterations observed in animals treated with PBBs suggest that these compounds modify endogenous steroid sex hormone metabolism to the extent that reproductive function is diminished. Female rats exposed perinatally to PBBs had delayed vaginal opening (Harris et al, 1978) and displayed lengthened estrous cycles (Johnson et al, 1980). Rhesus monkeys also displayed lengthened menstrual cycles which were associated with flattened and lengthened serum progesterone peaks (Lambrecht et al, 1978). Rhesus monkeys exposed to PBBs in the diet had hypoactive seminiferous tubules (Allen et al, 1978) and young bulls fed PBB had testicular atrophy and reduced spermatogenesis (Jackson and Halbert, 1974).

PBBs have been shown to attenuate the biological response to a variety of steroid hormones by increasing their microsomal metabolism. A marked reduction in the time of progesterone anesthesia in rats was noted concurrent with PBB induction of progesterone 16a-hydroxylation (McCormack et al, 1979; Arneric et al, 1980). The uterotropic action of exogenously administered estradiol and estrone was decreased by PBB treatment, and the increase in cytosolic estrogen receptors normally seen with estradiol administration was blunted (Bonhaus et al,1981).

Male reproductive dysfunction has also been found with PBB administration. PBB treatment has resulted in decreased seminal vesicle and prostate weights (Johnston et al, 1980), an effect which may have been mediated by increased hepatic testosterone metabolism. PBBs have been shown to increase oxidative testosterone metabolism in vitro in an age and sex dependant manner (Newton et al, 1982), and to decrease 5a-reduction of testosterone in both male and female rats. Decreased efficacy of reproductive steroid hormones due to increased metabolism is one possible mechanism for the reproductive deficits seen in PBB treated animals. If increased metabolism of circulating steroids, particularly androgens, occurred during the critical period of sexual differentiation (see below) the reproductive deficits seen with PBB and with other MFO inducers could be permanent, due to effects on brain differentiation or decreases in cytosolic androgen receptors in peripheral reproductive tissues.

Reproductive Consequences of End-Organ Metabolism of Testosterone

The effects of reproductive hormones such as testosterone fall into one of two catagories:

1. Direct effects of testosterone on reproduction in normal or castrated adult animals, and

2. Effects of testosterone resulting from perinatal sexual differentiation or "imprinting" of target organs such as brain and secondary sex structures.

A number of effects seen in adult animals require perinatal sexual differentiation or "imprinting" by testosterone (or the absence of testosterone in the female). Testosterone imprinting often requires the metabolic activation of the androgen. Generally, target organ metabolism of testosterone forms one of two active species: 5a-dihydrotestosterone or estradiol (see Figure 1). 5a-Reduction of testosterone to form 5a-dihydrotestosterone (DHT) is an essential step required by some tissues such as the external genitalia before masculinization occurs. Even in tissues such as the Wolffian ducts, which are affected directly by testosterone, DHT exhibits greater potency (Bardin and Catterall, 1981). Aromatization, on the other hand, is required for the differentiation of the mammalian brain to establish appropriate male behavior patterns (MacLusky and Naftolin, 1981). Aromatization also plays a role in androgen action in some muscle tissue, notably the levator ani muscle (Bardin and Catterall, 1981).

Morphological differentiation relies on the presence of testosterone during the critical period of sexual differentiation (days 17 to 28 post-conception in the rat). Fetal testis, under the control of the fetal

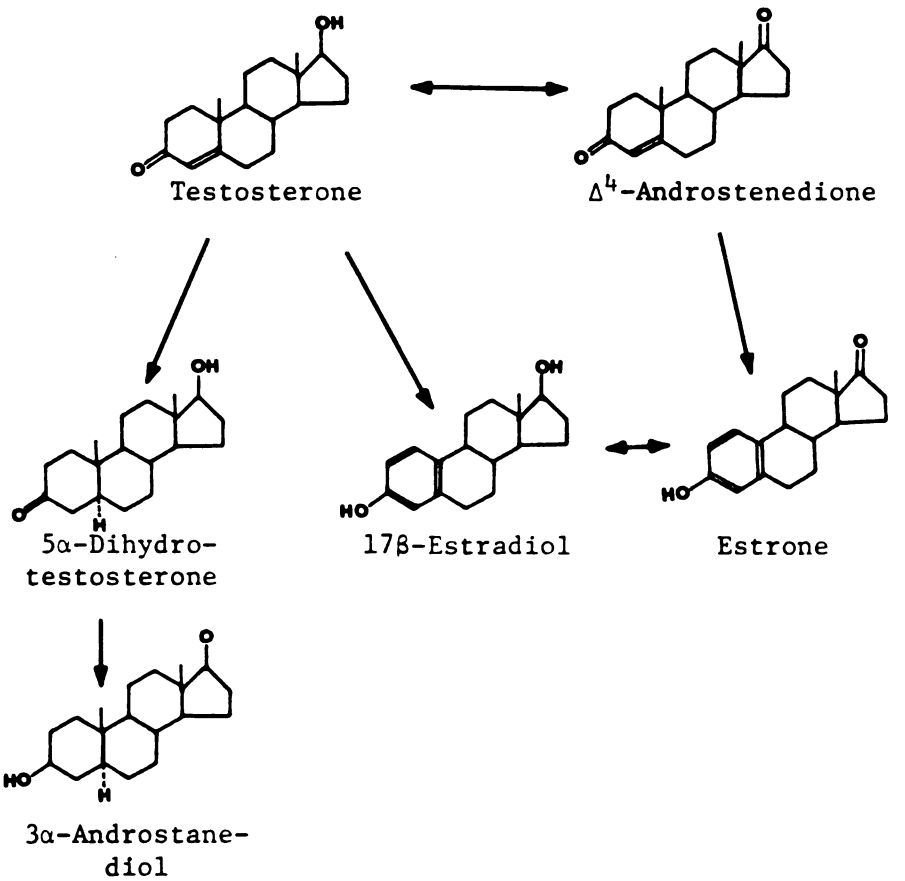

FIGURE 1. Formation of active metabolites of testosterone.

pituitary and/or the placenta (Wilson, 1981) secrete at least two organizing hormones needed for male differentiation. The first hormone is known as Mullerian Regression Factor (MRF), and is a peptide hormone of approximately 70,000 MW. MRF causes the atrophy and regression of the female structures known as the Mullerian Ducts. The second hormone required for sexual differentiation is testosterone. In the absence of MRF and testosterone the fetus develops as a morphological female. Unlike MRF, testosterone is a steroid which must undergo metabolism in at least some target tissues for sexual differentiation to be complete. Defects in testosterone synthesis result in a condition known as female pseudo-hermaphroditism, a condition characterized by undescended testes, absence of male external genitalia, and feminine psychosocial orientation. Defects in 5a-reduction of testosterone, while indistinguishable externally from testosterone deficiency, prevent development of external genitalia and the urogenital sinus, although normal development of internal male structures (Wolffian ducts) is allowed. In addition, 5a-reductase deficiency allows central male diffentiation to proceed normally via the aromatase pathway resulting in male sexual behavior patterns in rodents, and allows normal male behavior to develop in humans (MacLusky and Naftolin, 1981; Ehrhardt and Meyer-Bahlburg, 1981).

Brain sexual differentiation depends on the production of estrogens (17B-estradiol and estrone) from perinatal circulating androgens (testosterone and androstenedione). The aromatization reaction which results in the production of estrogens from androgens occurs in the brain regions affected (Naftolin et al, 1975; Naftolin et al, 1972; Selmanoff et al, 1977) and is believed to follow the same biochemical pathway seen in placental microsomes (see Figure 2) (Selmanoff et al, 1977). Briefly, the aromatizable androgen is hydroxylated at the 19-position, then further oxidized at the 19-carbon resulting in the formation of the gem-diol in equilibrium with the 19-oxo-androgen (Thompson and Siiteri, 1974; Braselton et al, 1974). The last hydroxylation reaction is the rate limiting step in aromatization, and results in the formation of 2B-hydroxy-19-oxo-androstenedione, followed immediately by the loss of the #19 carbon, the spontaneous collapse of the A ring into an aromatic configuration, and the reduction of the 3-keto group to a 3-hydroxy group (Goto and Fishman, 1977). The formation of these hydroxylated intermediates requires the presence of NADPH and molecular oxygen.

Central effects which rely upon aromatization of androgens to estrogens include the following: gonadotropin secretion (Worgul et al, 1981), prolactin secretion, learning, circadian rhythmns (MacLusky and Naftolin,

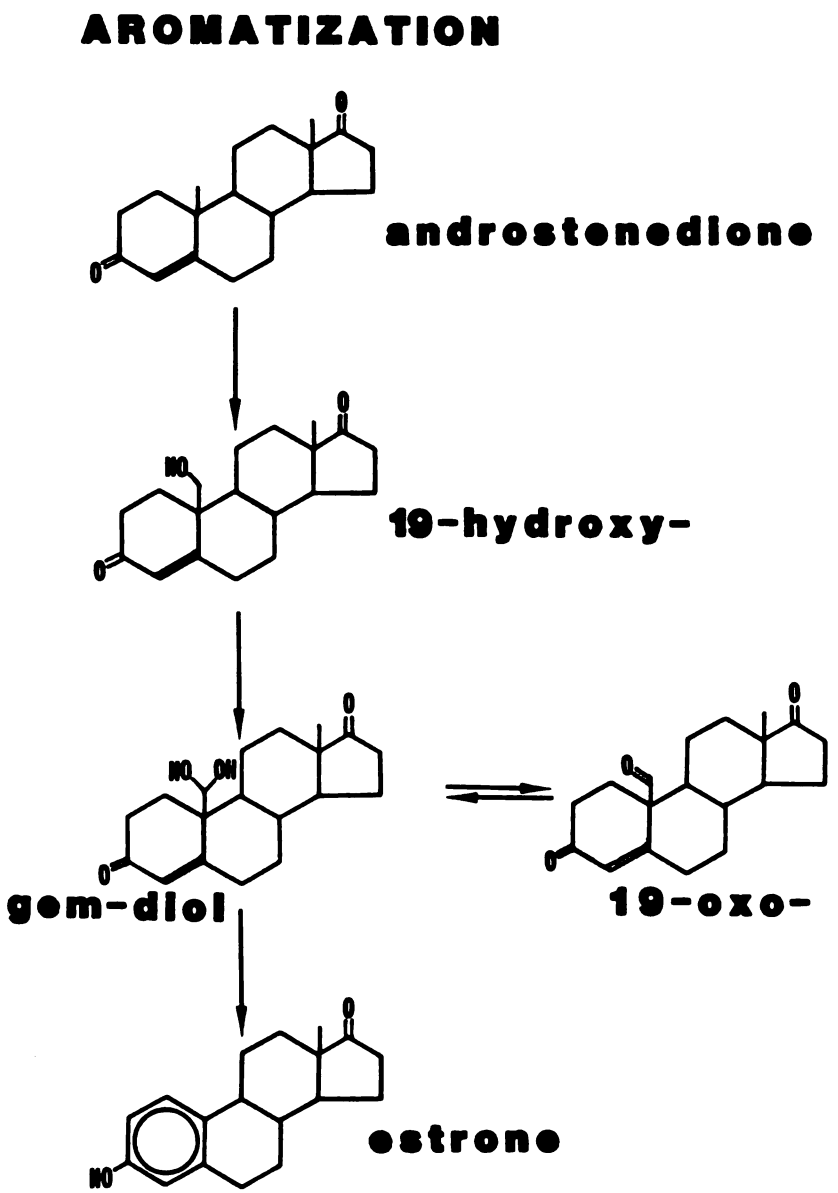

FIGURE 2. Intermediates formed in the aromatization of androstenedione.

1981), reproductive behaviors (Clemens and Pomerantz, 1982; Whalen and Olson, 1978), agression and play, and regulation of hepatic drug and steroid metabolism (Gustafsson et al, 1981). There is an extensive literature on the role which aromatization plays in adult as well as in perinatal brain (for list of references see MacLusky and Naftolin, 1981; Naftolin et al, 1975; and McEwen, 1982). In adult brain, aromatization regulates feedback control of gonadotropin secretion (Naftolin et al 1975, Worgul et al, 1981), and plays a role in the behavioral response to circulating or administered androgens (Clemens and Pomerantz, 1982; Whalen and Olson, 1978; Christensen and Clemens, 1975).

Aromatization of androgen to estrogen also results in differentiation of the neonatal brain. During the critical period of sexual differentiation, the neonatal brain is still plastic, and may be organized as a male brain by the administration of either estradiol or aromatizable androgen (MacLusky and Naftolin, 1981). Administration of 5a-reduced androgens alone is insufficient to cause masculinization of the neonatal brain, although 5a-reduction of testosterone may play some role in brain differentiation (Martini, 1982). A wide variety of experimental evidence confirms the important role of androgen aromatization for brain sexual differentiation. Perinatal castration results in

feminization of behavior and gonadotropin secretion patterns in male rats (Naftolin et al, 1975; McEwen, 1982; Whalen and Olson, 1981), testosterone or estradiol administration to either castrate male or female rats results in masculinization (Poplow and Ward, 1977; Whalen and DeBold, 1974), and administration of the estrogen antagonist MER-25 or the aromatization inhibitor androsta-1,4,6-triene-3,17 dione results in feminization of male fetuses (Naftolin et at, 1975; Clemens and Pomerantz, 1982; Whalen and Olson, 1981; Gladue and Clemens, 1980; Clemens and Gladue, 1978; Christensen and Clemens, 1975).

Aromatase activity in both male and female fetal brain has been shown to peak during the critical period of sexual differentiation (George and Ojeda, 1982), and testosterone titers in male rats also peak during the same time period (Orth et al, 1981; Weisz and Ward, 1980). Some researchers have shown that the developing brain is exquisitely sensitive to even a short-term drop in circulating testosterone titer (Ward and Weisz, 1980), and that androgen deprivation during the period of brain development will result in demasculinization and feminization of adult behavior (Ward, 1972).

Regulation of hepatic drug and steroid metabolism lies within the sexually differentiated region of the hypothalamus (Gustafsson, et al 1981). Influences which

feminize behavior also feminize hepatic steroid metabolic patterns. Control of hepatic steroid metabolism has been shown to reside in the pre-optic area of the hypothalamus (Gustafsson et al, 1981) the area with the maximal concentration of brain aromatase (Selmanoff et al, 1977), and the region of brain which is visibly sexually dimorphic (Gorski et al, 1978). A number of effects of steroids on hepatic metabolism have been shown to be mediated through the hypothalmic-pituitary axis, and to be dependant on brain aromatization of steroids (Einarsson et al, 1973; Gustafsson et al, 1981; Bardin and Catterall, 1981). The hypothalmic-pituitary axis, therefore, not only regulates reproductive physiology directly, but may further modulate reproductive events through control of hepatic steroid metabolism (Figure 3).

Experimental Hypothesis and Objectives

PBBs are potent inducers of mixed function oxidase enzyme systems, enzymes which are also responsible for the metabolism of endogenous steroid hormones. If PBBs decrease circulating steroid levels during the critical period of sexual development, impaired central differentiation may occur, with corresponding defects in central regulation of reproductive function. PBBs may produce some of their reproductive effects through

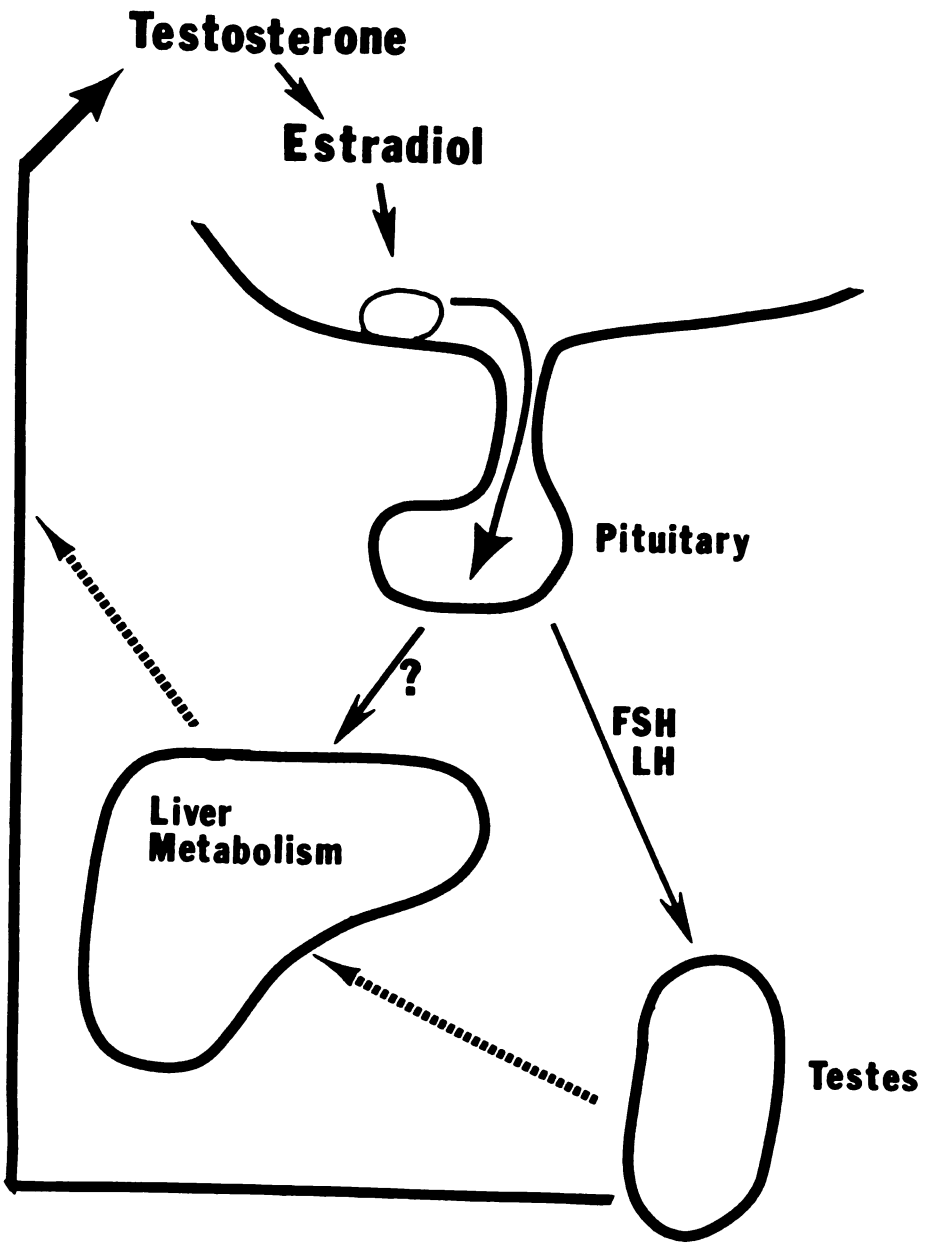

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FIGURE 3. Interactions between the hypothalamus/pituitary, gonadal steroid synthesis, and hepatic steroid metabolism.

alterations in central hypothalamic- pituitary control.

One indicator of the degree of brain differentiation is the activity of the hypothalamic aromatase enzyme. Research has established that females possess much lower levels of hyothalmic aromatase in adulthood than do males (Weisz and Gibbs, 1974), and treatments altering the neonatal or prepubertal endocrine environment might be expected to perturb the normal development of the aromatizing enzyme system. The hypothalamic aromatase, then, may be used as one indicator of reproductive function.

In order to use the hypothalamic aromatase as an indicator of central nervous system neuroendocrine function, an improved aromatase assay was needed. Previously available assay techniques are either too time consuming or insufficiently accurate (see Naftolin et al 1975; George and Ojeda, 1982).

In order to test our hypothesis that PBB induction of hepatic MFOs may alter central reproductive control through effects on hypothalamic aromatase the following experimental objectives were defined:

1. Develop a sensitive and reproducible assay for the determination of hypothalmic aromatase.

2. Determine normal levels of hypothalamic aromatase in male, female, and castrate male rats for use in experimental design of further studies on effects of PBBs

on central differentiation.

3. Determine the effects of pre-pubertal castration and PBB treatment on hypothalamic androgen aromatization.

4. Confirm the effects of polybrominated biphenyls on hepatic testosterone metabolism.

SECTION I

Assay Development

INTRODUCTION

In order to accurately determine the activity of the aromatase enzyme system in the hypothalamus, it was necessary to develop an improved assay. Although aromatizing activity is central to the normal control of reproduction, hypothalamic aromatase is active at very low levels, and radioactive tracer methods are necessary for its detection. In general, previous assays for aromatase have relied on one of two methods: production of $^{3}H_2O$ from the aromatization of 2-^{3}H-androstenedione (Thompson and Siiteri, 1974), or isolation and purification of ^{3}H-estrogens by solvent partition, thin layer chromatography, derivatization, and reverse isotope dilution followed by recrystallization to constant specific activity (Dessi-Fulgheri and Lupo, 1982; Selmanoff et.al., 1977; Naftolin et.al., 1972). Assays relying on the production of $^{3}H_2O$ do not always agree quantitatively with assays which detect directly the production of tritiated estrogen products (George and Ojeda, 1982). In addition, assays relying on the production of tritiated

water have been used primarily in tissues with higher aromatase activity than that reported in adult hypothalamus, and these assays may yield inaccurate results in the presence of non-aromatase enzymes capable of releasing tritium from 2-^{3}H-androgens (Hersey et.al., 1981). Although methods involving derivatization and multiple recrystallizations are more accurate, these techniques are time consuming and recoveries must be calculated using dual isotope techniques. These disadvantages rendered previously available techniques unsuitable, and high performance liquid chromatography (HPLC) seemed to hold the greatest promise of providing a rapid, sensitive and reproducible method. HPLC makes possible the determination of recovery by U.V. absorbance of reference standards added to the incubation extract, and offers the possibility of determining the production of several compounds in one chromatographic step. A valid enzyme assay would require the following conditions to be met: a) Incubation conditions which give time and protein dependant aromatization of the androgenic substrate, b) HPLC conditions which allow the rapid separation of the estrogenic products of aromatization, and c) proof of the radiochemical purity of the estrogens isolated by HPLC. Incubation conditions for placental and hypothalamic aromatase have been well described in the literature (Naftolin et.al., 1975; Selmanoff et.al., 1977; Ryan,

1959), but existing methods for determination of the reaction products are not suitable for detailed studies of the enzyme. The inital objective was therefore the development of a suitable HPLC system.

METHODS

Development of HPLC Systems

HPLC methods were developed on a Waters Assoc. chromatographic system consisting of a M-6000 pump, a model 440 U.V. absorbance detector set at 254nm, and a Waters 760 data module. Reference steroids were purchased from Steraloids Inc. (Wilton, N.H.), and dissolved in methanol for chromatography.

A variety of solvents were tested to determine which would adequately separate testosterone, androstenedione, estrone, and 17B-estradiol. Two solvent systems were developed for the assay, and two additional systems were used to confirm the purity of the peaks collected during the assay (Figure 4):

System A: acetonitrile/water (70:30), 1.0 ml/min, Waters 10μ radial-Pak C-18 cartridge;

System B: THF/water (35:65), 2.5 ml/min, Waters 10μ radial-Pak C-18 cartridge;

System C: acetonitrile/THF/water (55:5:40), 1.0 ml/min, Altex 5μ Ultrasphere ODS column (15 cm length);

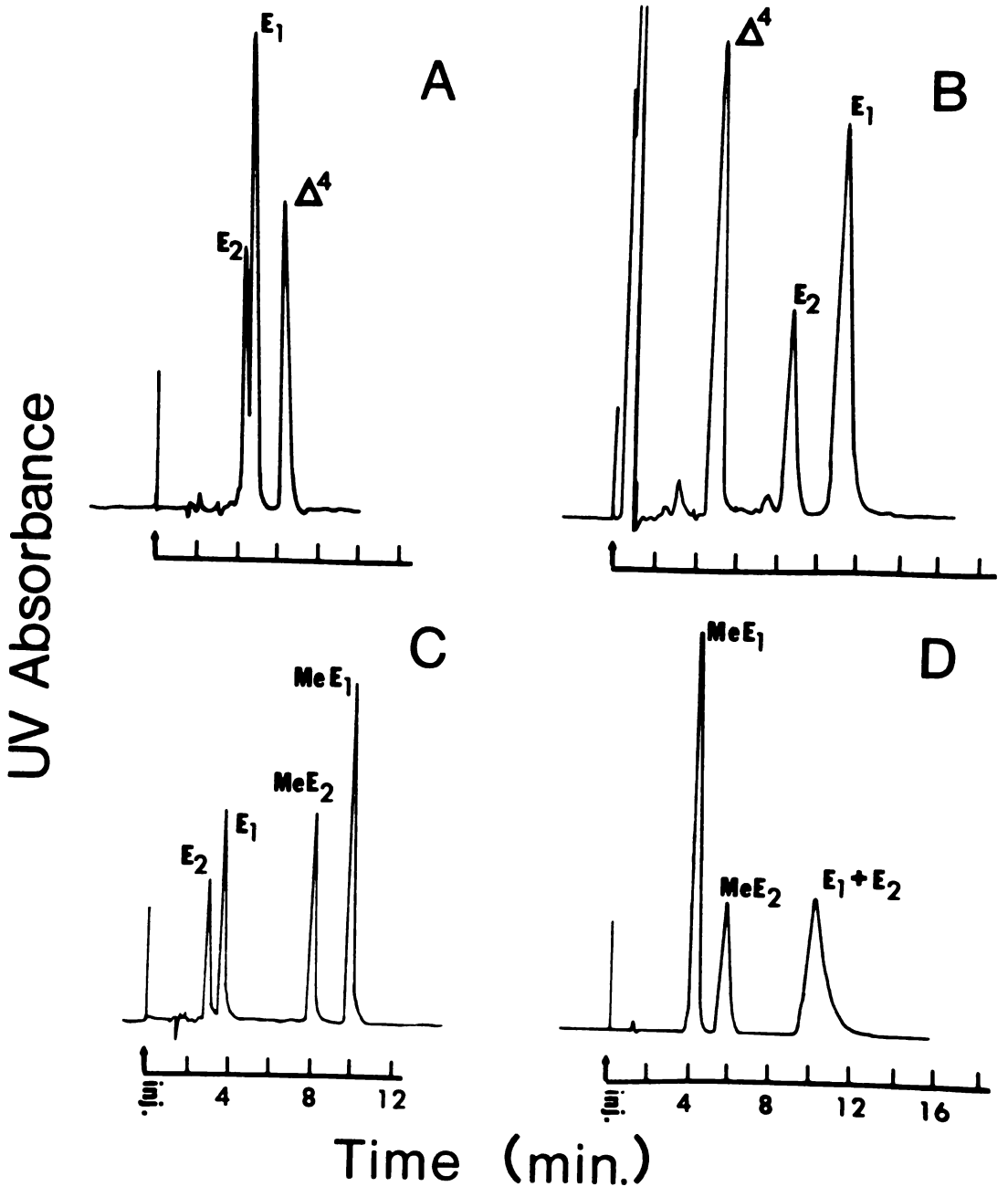

FIGURE 4. Chromatograms of reference steroids.
A) acetonitrile/water (70:30), C-18 column
B) THF/water (35:65), C-18 column
C) acetonitrile/THF/water (55:5:40), C-18
D) cyclohexane/n-octanol (95:5), normal phase

E_1=estrone, E_2=17B-estradiol, Δ^4= androstenedione, MeE_1=estrone-3-methyl ether, MeE_2=17B-estradiol-3-methyl ether.

System D: cyclohexane/n-octanol (95:5), 2.0 ml/min. Waters 10μ radial-Pak normal phase;

System A allowed the rapid separation of the products of aromatization (estrone and 17B-estradiol) from the tritiated androstenedione precursor. Since the estrogens elute before the androgen (Figure 4a), the chance of product contamination by the highly radioactive substrate was minimized.

System B, using THF and water, exhibited an entirely different pattern of solvent selectivity. Chromatograms of reference steroids (Figure 4b) show that the estrogens eluted behind testosterone and androstenedione. Because of the differences in selectivity exhibited by acetonitrile and THF, it is unlikely that a compound co-eluting with one of the estrogens on system A would also co-elute on system B. Therefore, preliminary chromatography of a sample on system A with collection of the estrogen fractions, followed by a second chromatographic purification of the collected fraction on system B, should yield estrogen peaks of high purity.

Systems C and D (Figure 4c and 4d) were developed to separate the 3-methyl ethers of estrone and estradiol from androstenedione and the parent estrogens. These systems were used to confirm the purity of the estrone peak collected during chromatography on system B.

Preliminary Studies With Placental Microsomes

Placental microsomes were used as a source of aromatase for the initial experiments, since placenta has an active, well characterized aromatizing enzyme system (see Ryan, 1959; Braselton et.al., 1974). A healthy, fullterm human placenta was obtained from Sparrow Hospital (Lansing MI), and placental microsomes were prepared according to the method of Ryan (Ryan, 1959). The placenta was minced, the vascular tissue dissected away, and segments of the placenta were placed into cold (4° C) 0.05M phosphate buffer and homogenized in a Waring blender. The placental homogenate was centrifuged at 10,000 x g for 20 minutes, and the supernatant was drawn off, placed into ultacentrifuge tubes, and centrifuged at 105,000 x g for 60 minutes. The microsomal pellet was resuspended in 200mM sucrose/ 0.05M phosphate buffer at a dilution such that 1 ml of microsomes was equivalent to 10 g of tissue. The microsomes were then stored at -70° C until used.

Placental microsomes equivalent to 1 g of tissue were incubated in 0.05M phosphate buffer (pH=7.0) at 37° C for one hour in a total volume of 1 ml containing 40μM ^{14}C-testosterone (25μCi/μmole), and an NADPH regenerating system consisting of 2.4 μM NADP, 11.2 μM glucose-6-phosphate, and 0.4 U glucose-6-phosphate dehydrogenase. Following incubation for one hour, the reaction was terminated by the addition of 5 ml of

extracted twice with methylene chloride. The combined methylene chloride extracts were evaporated to dryness under N_2, and partitioned between 1.0N NaOH and toluene. The NaOH layer was neutralized by the addition of 5N HCl, and extracted twice with methylene chloride. The methylene chloride extract was evaporated to dryness under N_2, and dissolved in 0.5 ml of methanol for chromatography.

Chromatography of the placental incubation extracts resulted in radiochromatograms showing good separation of the ^{14}C-estrogens produced from the ^{14}C-testosterone precursor (Figure 5a and 5b). Both system A and system B show the estogens adequately separated from testosterone, and measurable but minimal 17B-oxidation of estradiol to estrone.

Purification of Tritiated Androstenedione

Following the successful incubation of placental microsomes with ^{14}C-testosterone, hypothalamic incubations under the same conditions were attempted using (1,2,6,7)-^{3}H-androst-4-ene-3,17-dione (90 Ci/mmole) as the precursor. These initial experiments with hypothalamic tissue failed to yield tritiated estrogens above those seen as "background" in incubation blanks. Because of the high background in the substrate, two purification steps, corresponding to chromatography of the ^{3}H-androstenedione on system A and then on system B were used to purify

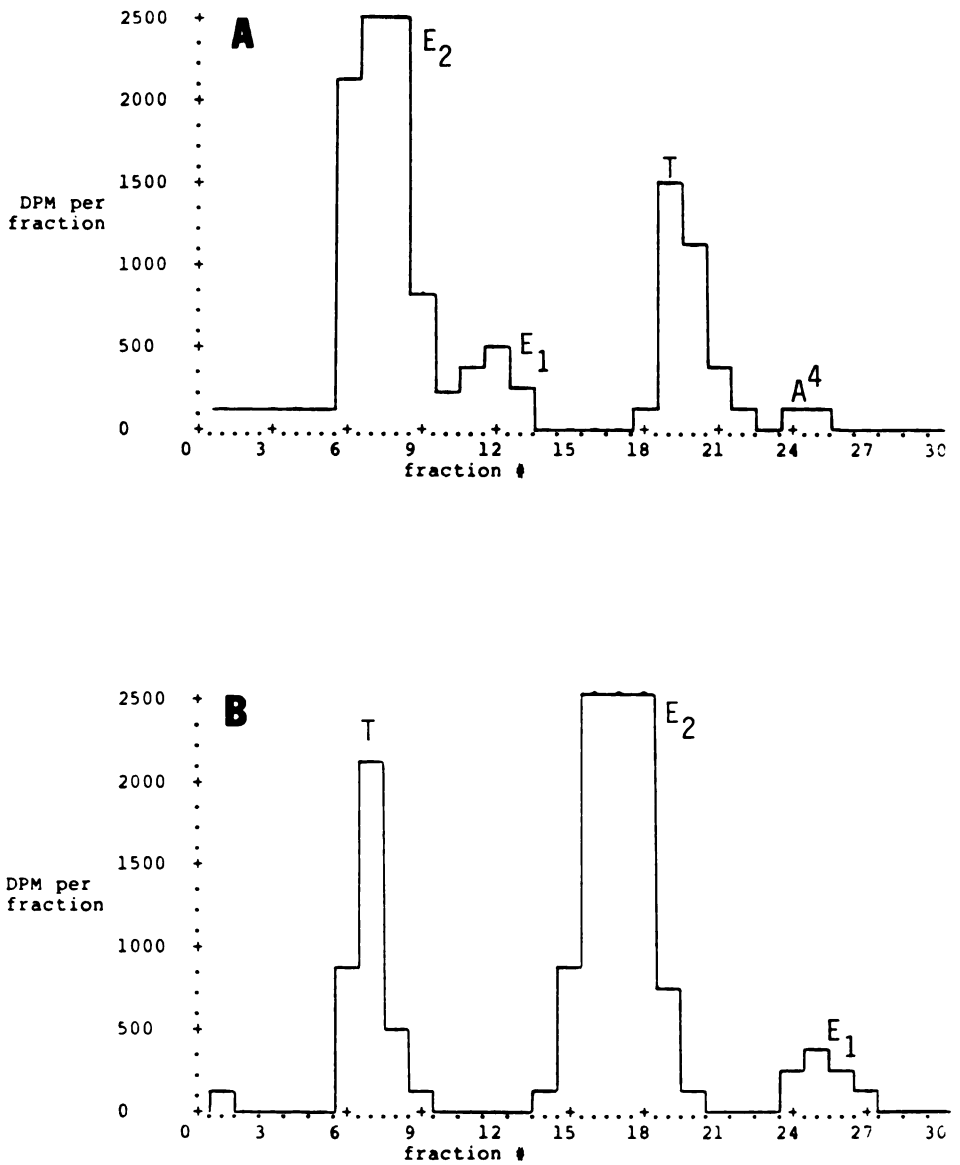

FIGURE 5. Radiochromatogram of products of incubation of placental microsomes with ^{14}C-testosterone.
A) chromatography on system A
B) chromatography on system B

E_1=estrone, E_2=17B-estradiol, T=testosterone, A^4=androstenedione.

the substrate before incubation. Repeated hypothalamic incubations still failed to yield tritiated estrone, although incubations of placental microsomes did produce the expected estrogens.

Multiple rechromatographies of the ^{3}H-androstenedione using systems similar to HPLC systems A and B disclosed the presence of androsta-1,4,6-triene-3,17-dione present as an impurity. The synthesis of the tritiated androstenedione by New England Nuclear, the supplier, used androsta-1,4,6-triene-3,17-dione as the starting material. Since the 1,4,6-triene has been shown to be a potent aromatase inhibitor (Schwartzel and Brodie, 1973), its presence was suspected of inhibiting the hypothalamic aromatase. The purification steps employed initially (chromatography on system A followed by chromatography on system B) had failed to remove the 1,4,6-triene because the compound co-eluted with androstenedione on both systems.

An improved purification technique was therefore developed which used chromatography on system B to remove estrogens present as impurities, followed by chromatography of the androstenedione peak on a system with 40% acetonitrile as the eluent. Chromatography of the androstenedione with 40% acetonitrile removed all traces of 1,4,6-triene present.

Incubation of Hypothalamic Tissue

Adult male Sprague-Dawley rats were killed by decapitation, and the entire brain removed and frozen on dry ice. Anterior hypothalamus containing the median pre-optic area was removed using a dissection similar to that reported by Naftolin (Naftolin et.al., 1972). The median pre-optic area has been shown to contain greater than 2/3 of the brain aromatase (Selmanoff et.al., 1977). The dissected hypothalamic tissue was homogenized in 0.05M phosphate buffer (pH=7.0), and microgram quantities of estradiol and estrone were added to allow easy calculation of recovery following incubation. The use of cold estrone and estradiol in the incubation was suggested by Naftolin (Naftolin et.al., 1975) as a technique to prevent further metabolism of ^{3}H-estrogens produced by aromatiation. In this case, the estrogen "trap" also functioned to allow easy calculation of recovery. This technique was possible because estrogens do not "product inhibit" the aromatase enzyme (Naftolin et.al., 1975; Schwarzel and Brodie, 1973).

Each assay tube contained hypothalamic homogenate (5 - 10 anterior hypothalami), 200nM ^{3}H-androstenedione (90 Ci/mmole) and an NADPH regenerating system consisting of 2.4μM NADP, 12μM glucose-6-phosphate and 0.4 U glucose-6-phosphate dehydrogenase in a total volume of 0.5 ml. The reaction was started by the addition of the

^{3}H-androstenedione and the assay tubes were incubated with agitation for 3 hours at 37° C. The reaction was terminated by the addition of 5 ml of methylene chloride. Blanks were prepared by the addition of methylene chloride at zero time, or by incubation of boiled cerebral cortex homogenates for 3 hours.

Each incubation tube was extracted twice with 5 ml of methylene chloride, the combined extracts were dryed over anhydrous Na_2SO_4 and evaporated to dryness under N_2. Each sample was dissolved in 0.5 ml of methanol/ water (90:10), and extracted with 1.0 ml of hexane to remove non-polar lipids. The methanol/water layer containing the estrogens was evaporated to dryness and redissolved in 50 µl of methanol prior to chromatography.

The samples in methanol were chromatographed on HPLC system A, and the estrone and estradiol fractions collected for rechromatography. The estrone peak was again collected during chromatography on system B, and the ^{3}H-estrone present was quantified by liquid scintillation counting in a Packard Tri-Carb, (Model 460 C). Recovery of estrone was calculated from U.V. absorbance during chromatography on system B.

Method Validation

Several test incubations were performed in order to verify the purity of the estrone peak collected and counted following chromatography on system B. The first incubation was carried out in 6 tubes, each containing a different amount of placental microsomes. After incubation as described above, the estrogenic products in each tube were subjected to chromatography using system A, then split into two aliquots. The first aliquot was chromatographed on system B, and the specific activity of the estrone collected was determined. The second aliquot was run on system B, the estrone peak collected, and then methylated by the method of Brown (Brown, 1955). The 3-methyl ether of estrone was then chromatographed on system C, and the specific activity of the estrone methyl ether compared to the specific activity of the estrone obtained during chromatography on system B.

Peak purity was also checked for incubations containing homogenates of hypothalamic tissue. Three tubes, each with 10 hypothalami, were incubated as described above. Peak purity of the estrone produced following chromatography on system B was determined as described above for placental microsomes, however following methylation the specific activity of the estrone methyl ether produced was checked on both system C and system D. In addition, time and protein dependancy of the hypothalamic incubations

were verified using pooled homogenate equivalent to two to eight hypothalami, and for times up to 3 hours.

RESULTS AND DISCUSSION

Chromatography with system A was useful in separating the estrone produced from the highly radioactive ^{3}H-androstenedione substrate. Since the estrogens eluted first on system A, the chance of the androstenedione "tailing into" the estrogen peaks was eliminated. The second chromatographic step with system B then separated the estrogens from each other, and from any residual androstenedione which might remain.

Estrone recovered following chromatography with system B averaged 70 - 80 % of the estrone added to the incubation tubes. Figure 6 shows a radiochromatogram (HPLC system B) of the estrone produced by incubation of ^{3}H-androstenedione with hypothalmic tissue in comparison with that seen in a blank incubation. Since hypothalamic tissue has been shown to contain little 17B-dehydrogenase (George and Ojeda, 1982), and since only trace amounts of estradiol were observed following incubation of hypothalmic homogenates (Figure 6), the estradiol peak was not further characterized, and was not used in the calculation of hypothalamic aromatizing activity.

Chromatography on system B produced estrone with an

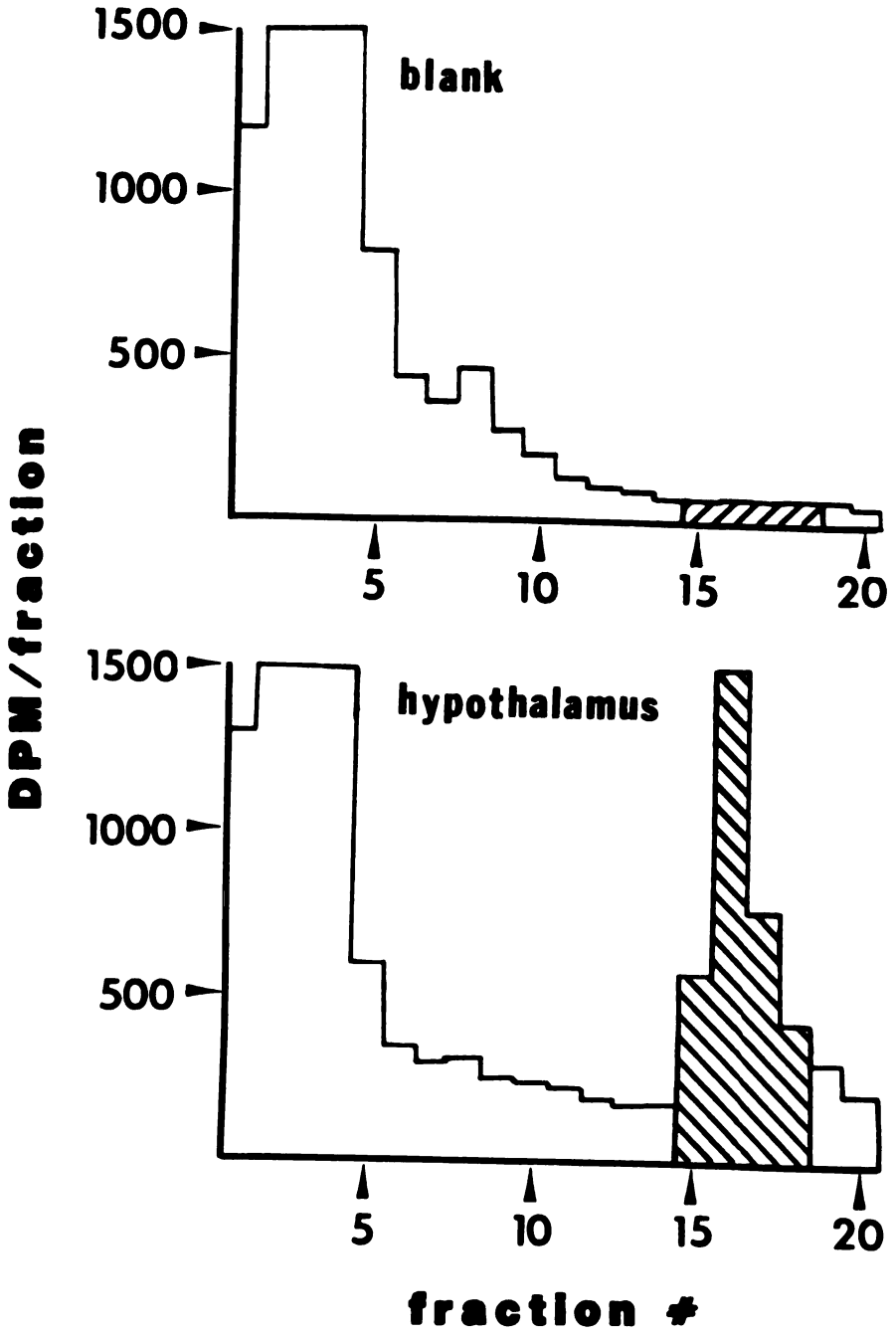

FIGURE 6. Radiochromatogram of fractions collected from system B chromatography of zero-time blank and hypothalamic homogenate incubation. Fractions collected every 30 seconds beginning at 4 minutes. Cross-hatch pattern corresponds to extrone peak with U.V. detector (280 nm).

TABLE 1.
Purity of Estrone Produced by Aromatization Following Reverse Phase Chromatography with 35% THF.

sample #	spec. activity after THF dpm/µg estrone	spec. activity following methylation reverse phase dpm/µg*	%THF	normal phase dpm/µg*	%THF
Hypothalamic homogenate					
1	213	224	105.%	221.	103.%
2	452	411	95.6%	371	86.3%
3	266	242	95.6%	247	97.6%
			98.7±3.1		95.6±5
Placental microsomes					
1	119	118	99.2%		
2	1050	1090	104. %		
3	793	752	94.8%		
4	191	174	91.1%		
5	686	684	99.7%		
6	402	363	90.3%		
			96.5±2.4		

* calculated on the basis of µg of original estrone added.

average purity of 95% or greater (Table 1). This was shown by collection of the estrone fractions from system B, methylation, and rechromatography on system C or D. Since the criterion for purity using recrystallization is normally a 5% or smaller change in isotope ratio, the purity of estrone produced by this method is comparable to that produced by repeated recrystallization.

Both time and protein dependancy were initially determined using placental microsomes (data not shown), and confirmed using hypothalmic incubations. This incubation technique showed linear time dependancy to 3 hours, and was protein dependant with hypothalamic protein equivalent to 2 to 8 hypothalami (Figure 7).

These results indicate that hypothalamic aromatase may be measured following separation of estrogens on HPLC system A and isolation and quantification of estrone on HPLC system B. Whereas previous methods have offered recoveries of around 50% (Weisz and Gibbs, 1974), this method offers recoveries of 70 - 80%, and is more rapidly performed than previous techniques which have relied on solvent partition, thin-layer chromatography, derivatization, and reverse isotope dilution with multiple recrystallizations. This method offers isolation of products of high (95% or greater), proven purity and it is sensitive enough to allow the detection of less than 10 femtomoles of tritiated estrogen produced by aromatization.

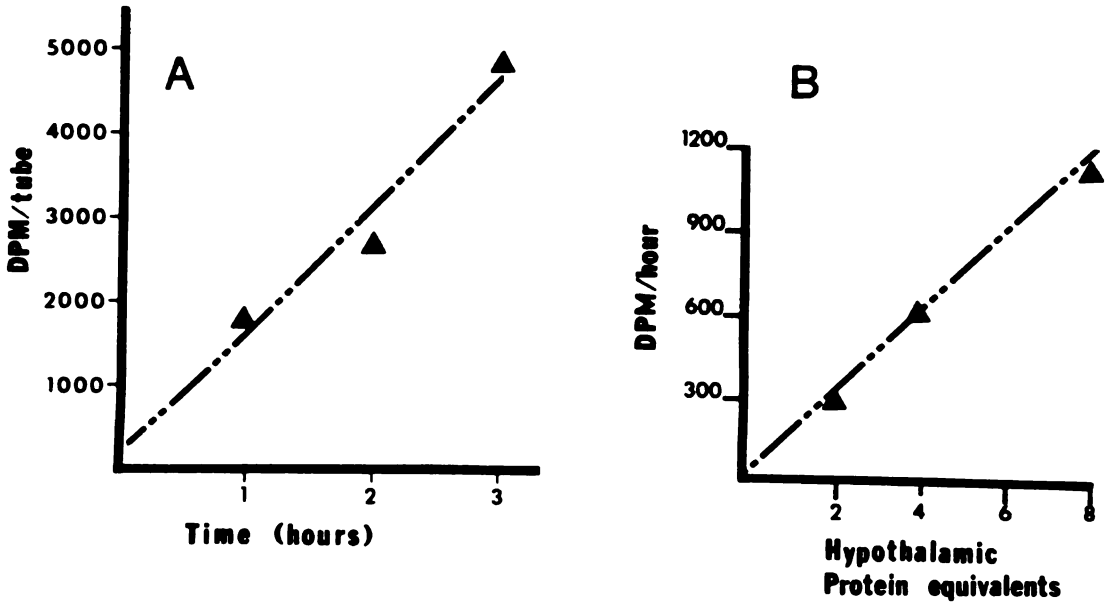

FIGURE 7. Time (A) and protein dependence (B) of incubation of hypothalamic homogenates.

SECTION II

Effects of Orchidectomy or PBB Treatment on Hepatic Testosterone Metabolism

INTRODUCTION

Hepatic oxidative metabolism of testosterone acts to produce physiologically less active forms of this androgen. These compounds then readily undergo phase II metabolism and are excreted. Increases in sex steroid metabolism may be expected to result in impaired reproductive function if homeostatic mechanisms are unable to compensate. Polybrominated biphenyls are known potent inducers of hepatic oxidative metabolism (Dent, 1978; Dent et.al., 1976; McCormack et.al., 1978), and have been shown to specifically induce the hepatic mixed-function oxidase system responsible for oxidative metabolism of steroids (Nowicki and Norman, 1972; Arneric et.al. 1980; Newton et.al., 1982). In vivo studies have shown that PBBs reduce the efficacy of exogenously administered steroids. PBBs decrease the uterotropic action of exogenously administered estradiol (Bonhaus et.al., 1981), and reduce the time of progesterone induced anaesthesia in rats (McCormack et.al., 1979).

PBBs have produced a variety of endocrine and reproductive effects which may be linked to increased microsomal metabolism of sex steroids. PBB fed bulls have

shown testicular atrophy and decreased spermatogenesis (Jackson and Halbert, 1974; Kimbrough et.al., 1978). Male rats treated perinatally with PBBs showed decreased prostate and seminal vesicle weights, and female rats displayed lengthened estrous cycles (Johnson et al, 1980). In addition, the hypertrophic response of rat seminal vesicle to exogenous testosterone was decreased (McCormack et al, 1979).

Polybrominated biphenyls are "mixed" inducers of hepatic microsomal enzymes, having the inducing properties of both P-450 inducers such as phenobarbital, and P-448 inducers such as 3-methylcholanthrene. Although the individual PBB congeners have been shown to possess either P-450 or P-448 inducing activity, the PBB mixture, Firemaster BP-6, contains congeners which induce both P-450 and P-448 (Dent, 1978).

PBB induction of hepatic testosterone metabolism has been shown to follow a pattern which is dependent on both the age and the sex of the animal treated (Newton et al, 1982). The sex difference in MFO induction may be related to perinatal imprinting of steroid metabolism which occurs under the influence of circulating testosterone (Skett and Gustafsson, 1979; Einarsson et al 1973; Gustafsson et al, 1981). Since circulating steroid levels also affect hepatic steroid metabolism directly, changing steroid levels during sexual maturation of the animals may also

play a role in the age difference in response to PBBs.

The objective of this study was to examine the effects of acute PBB treatment on hepatic testosterone metabolism, and to compare male and female testosterone metabolic patterns with those seen in PBB induced and orchidectomized animals.

METHODS

Animals and Treatment

Weaned, 21 day old male and female Sprague-Dawley rats were obtained from Spartan Farms (Haslett, MI). The animals were randomly assigned to one of five treatment groups according to sex. The treatment groups were as follows:

1. control male;
2. control female;
3. PBB treated males, given 150 mg/kg of Firemaster BP-6 (i.p. in peanut oil) at 22 days of age;
4. PBB treated females, 150 mg/kg Firemaster BP-6 (i.p. in peanut oil) at 22 days of age;
5. males castrated at 22 days of age.

Each treatment group contained 15 to 17 animals. The animals were maintained on a 12 hour in phase light:dark cycle, with water and food (Wayne Lab Blox) available ad libitum until sacrifice at 75 days of age.

Preparation of Liver Microsomes

Animals were killed by decapitation, and the livers immediately removed into ice-cold 1.15% KCl. The livers

were then weighed, and homogenized in 4 volumes of 66mM Tris buffer using a glass and teflon Potter-Elvehjem homogenizer. The homogenate was centrifuged at 10,000 x g for 20 minutes, the supernatant drawn off and recentrifuged at 105,000 x g for 60 minutes. Following ultracentrifugation, the microsomal pellet was re-suspended in 66mM Tris/ 200mM sucrose buffer and frozen at -70° C until used. Microsomal protein concentrations were determined with a modification of the Lowry method (Lowry et.al., 1951) using bovine serum albumin as the standard.

Determination of In Vitro Testosterone Metabolism

Hepatic oxidative testosterone metabolism was measured using the method of Newton (Newton et.al., 1982). Each incubation tube contained 0.8 mg of microsomal protein, 115 umole testosterone, and an NADPH regenerating system consisting of 0.3 µmole NAD, 0.7 µmole NADP, 5.8 µmole glucose-6-phosphate and 1 U of glucose-6-phosphate dehydrogenase in a total volume of 1.06 ml. After preincubation for 5 minutes the reaction was started by the addition of testosterone. The incubation mixtures were maintained at 37° C for 30 minutes with agitation.

Reactions were terminated by the addition of 5 ml of chloroform/diethyl ether (3:1). Each incubation tube was extracted 3 times with 5 ml of the chloroform/ether

mixture, and the extract dryed by passage over anhydrous Na_2SO_4. The dryed extracts were evaporated to dryness under N_2, resuspended in 1.0 ml ethanol, and filtered prior to chromatography.

High Performance Liquid Chromatography

The oxidative metabolites of testosterone were quantified by the method of Newton (Newton et.al., 1982). The testosterone metabolites 7a-hydroxytestosterone, 16a-hydroxytestosterone, 6B-hydroxytestosterone, and androstenedione were separated and quantified by HPLC. The chromatography system consisted of a Waters M-6000 pump, 441 detector (254 nm), 720 system controller, Waters Intelligent Sample Programmer (WISP), and Waters 760 data module. The column used was an Altex 5μ Ultrasphere ODS column, with 24% THF as the eluent at a flow rate of 1.0 ml/min. Metabolites were quantified by external standard quantitation using known reference steroids (Steraloids, Wilton N.H.) as standards.

Statistical Analysis

Metabolite concentrations were determined by integration of peak areas using the Waters 760 data module. Data were analyzed using a completely random analysis of variance (a=0.05) for each metabolite, followed by Duncan's New Multiple Range Test (a=0.05) for comparison of treatment means.

RESULTS

Sex differences in testosterone oxidation

Control male rats showed greater 16a-hydroxylation, 6B-hydroxylation, and 17B-oxidation than did control females (Figure 8b, 8c, 8d and Table 2). Control females, however, had increased production of 7a-hydroxytestosterone when compared with control male animals (Figure 8a).

Effect of PBB treatment

PBB treatment increased the production of 7a-hydroxytestosterone in microsomes from both male and female rats, and increased the production of 16a-hydroxytestosterone and 6B-hydroxytestosterone in male but not in female rats (Figure 8a, 8b, 8c and Table 2). PBB treated males exhibited 7a-hydroxytestosterone levels comparable to those seen in control females. Androstenedione production was unaffected by PBB treatment in male rats, but was increased by PBB treatment in female rats (Figure 8d).

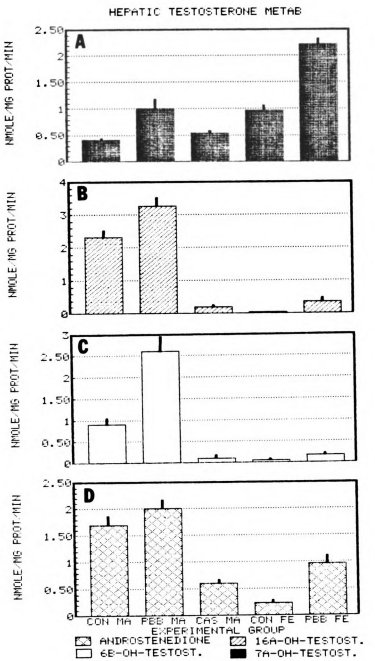

FIGURE 8. Products of oxidative testosterone metabolism in vitro by livers of orchidectomized, control, or PBB treated rats.
A) 7a-hydroxytestosterone
B) 16a-hydroxytestosterone
C) 6B-hydroxytestosterone
D) androstenedione

CON MA=control male, PBB MA=PBB treated males, CAS MA=castrate males, CON FE=control females, PBB FE= PBB treated females.

TABLE 2
Products of oxidative testosterone metabolism *in vitro* by livers of orchidectomized, control, or PBB treated rats.

group	7a-hydroxy-testosterone	16a-hydroxy-testosterone	6B-hydroxy-testosterone	androstene-dione	total metabolites
control male	.405±.04(7)[a]	2.34±.21(7)	.923±.10(7)	1.71±.20(7)[a]	5.38±.43(7)
PBB male	1.01±.14(7)[b]	3.26±.24(7)	2.60±.21(7)	2.01±.21(7)[a]	8.88±.60(7)
castrate male	.539±.04(7)[a]	.201±.07(7)[a]	.118±.04(7)[a]	.598±.21(7)[b,c]	1.46±.31(7)[a]
control female	.971±.10(8)[b]	.032±.01(8)[a]	.055±.01(8)[a]	.236±.05(8)[b]	1.29±.15(8)
PBB female	2.22±.15(10)	.368±.06(10)[a]	.167±.02(10)[a]	.971±.17(10)[c]	3.73±.26(10)

all measurements are nmole/mg protein/min, expressed as mean±standard error (n)
a,b,c letters denote values which are not statistically different from other values designated with the same letter (within columns only).

Effect of castration on testosterone metabolism

The effect of castration was to reduce total testosterone metabolism to levels seen in normal female rats (Figure 9 and Table 2). Although castration failed to change the level of 7a-hydroxylation when compared to male controls, orchidectomized males produced levels of 16a-hydroxy- testosterone, 6B-hydroxytestosterone, and androstenedione similar to those seen in the females tested (control and PBB treated females).

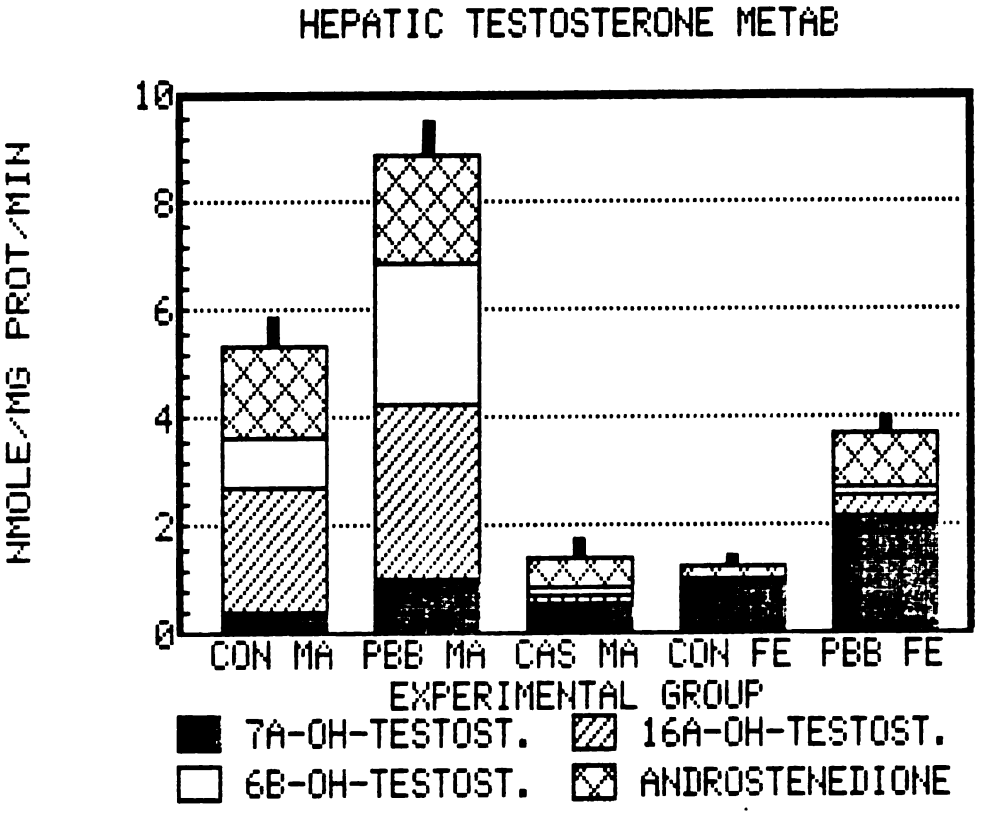

FIGURE 9. Total testosterone metabolism <u>in vitro</u> by liver microsomes from orchidectomized, control, or PBB treated rats.

CON MA=control male, PBB MA=PBB treated males, CAS MA=castrate males, CON FE=control females, PBB FE=PBB treated females.

DISCUSSION

A summary of total testosterone metabolism (Figure 9) reveals that in general, a single dose of PBB (150 mg/kg) was sufficient to induce testosterone metabolism for at least 55 days following treatment in both male and female rats. Castrate males showed total testosterone metabolism similar to that seen in control females, indicating that testosterone supports its own metabolism.

Since differences in metabolism were first linked to the sex of animals (Nicholas and Barron, 1932), experiments which attempted to define the role of the sex steroids in modulating metabolism have been numerous. Neonatal castration of male rats altered hepatic metabolism to a pattern resembling that seen in female animals, although adult castration had variable effects on the pattern of drug metabolism (Einarsson et al., 1973). Castration before 4 weeks of age has been shown to result in feminization of hepatic metabolism (Kato, 1974). The present experiment clearly demonstrated a castration induced feminization of 16a- and 6B- hydroxylation and androstenedione production, while 7a-hydroxylation was unaffected.

PBB treatment resulted in induction of total testosterone metabolism. Male animals showed induction of the P-450 linked enzymes 16a- and 6B- hydroxylase, while female animals exhibited marked increases in 7a-hydroxylation. Testosterone 16a-hydroxylase activity is sexually differentiated by circulating androgens during neonatal development, whereas 6B-hydroxylase activity is under direct androgenic control (Einarsson et al, 1973). Testosterone 7a-hydroxylase is regulated by non-hormonal factors in adulthood, and shows both higher basal activity as well as greater inducibility in female rats (Einarsson et al, 1973).

Newton et al (1982) have previously demonstrated that chronic PBB treatment results in induction of hepatic steroid metabolism. The results of this study are in general agreement with those reported by Newton, although some differences in the exact pattern of enzyme induction were seen. These differences are attributable to the differences between the single dose treatment used in this study, the chronic dietary intake used by Newton, and age differences at time of treatment and sacrifice.

From the above results, it is apparent that even a single dose of PBB is capable of marked induction of testosterone metabolism. Whether the increased metabolism of steroids is related to reproductive effects associated with PBB treatment is of current interest. Decreased

testosterone levels during the perinatal period of sexual differentiation could lead to permanent reproductive deficits. Gupta et al (1982) have already shown that perinatal treatment with phenobarbital, a short-term inducer of steroid metabolism, is capable of eliciting a permanent decrease in circulating testosterone levels. Increased testosterone metabolism during the critical period of brain differentiation could permanently alter reproductive function in both male and female animals. PBB, a potent inducer of mixed function oxidases, should therefore be tested for effects on brain differentiation and neuroendocrine modulation of reproductive function.

SECTION III

Effect of Orchidectomy or Acute PBB Treatment on Hypothalamic Aromatase Activity

INTRODUCTION

The aromatization of androgens to estrogens in the central nervous system is a key step in the sexual differentiation of the brain, and the consequent sex differences seen in adult sexual behavior and physiology. In man (Naftolin et al, 1971) as in the rat (Naftolin et al, 1975; Weisz and Gibbs, 1974), circulating androgens from the fetal testis are aromatized into estrogens in the anterior hypothalamus, and direct the organization of the brain to the male pattern. Any interruption of this process either through reduction in circulating testosterone titers (Orth et al, 1981; Ward and Weisz, 1980), or blockade of the aromatase pathway in the hypothalamus (Gorski et al, 1977; Whalen and Olson, 1981), results in feminization of adult physiology and behavior (Ward, 1972; Gorski et al, 1978; Ward and Weisz, 1980).

Aromatization of testosterone and androstenedione to their corresponding estrogens, 17B-estradiol and estrone, also plays a key role in the regulation of adult reproductive physiology and behavior. Worgul et al (1981) have shown that in the dog, as in the rat, central aromatization is responsible for regulation of LH

secretion in the male. Other investigators have conjectured that it is androgen aromatization rather than circulating estrogens that modulate hypothalamo-pituitary feedback mechanisms in the female (Selmanoff et al, 1977).

Because of the key role played by hypothalamic aromatase in sexual differentiation of the brain and in adult neuroendocrinology, any alteration in this enzyme system could have far reaching reproductive consequences. PBBs have been shown to be potent inducers of the hepatic mixed-function oxidases which metabolize circulating steroids (Dent, 1978; Newton et al, 1982). Induction of MFOs may reduce circulating steroid levels, and thus alter hypothalamic aromatization, consequently affecting reproductive performance.

Although the presence of aromatase has been verified in the brain tissue of a variety of species (Naftlin et al, 1975; Clemens and Pomarantz, 1982; Sholl et al, 1982; Worgul et al, 1981; Reddy et al, 1973; Callard et al, 1978), little is known about its specific interactions with normal and perturbed endocrine systems. Early research demonstrated that rabbit aromatase could be influenced by castration or hormone treatment in adult animals (Reddy et al, 1973). The rabbit data is interesting, but since the rabbit is a reflex ovulator, the data have little predictive value in rat or human systems.

Because PBBs may alter the fetal endocrine environment by induction of steroid metabolism, PBBs could affect the hypothalamic aromatase system. Since PBBs have known reproductive effects, some of which may be mediated through central pathways, it is important to examine the hypothesized interaction of PBBs with hypothalmic aromatase. The following experiments were performed to determine the effect of PBB treatment on hypothalamic aromatase, and compare the effect with that produced by orchidectomy.

METHODS

Animals and Treatment

Weaned, 21 day old male and female Sprague-Dawley rats were assigned to PBB treated, control, or orchidectomized groups as described previously. Animals were maintained on a 12 hour light cycle with food and water available ad libitum until sacrifice at 75 days of age.

Assay of hypothalamic aromatase

At 75 days of age, animals were killed by decapitation and the brain was immediately removed onto dry ice. The brain was stored for 24 hours at -70° C until assayed for hypothalamic aromatase activity. (1,2,6,7)-^{3}H-Androst-4-ene-3,17-dione (New England Nuclear, Boston MA), was purified by two successive HPLC procedures as previously described (section I) to insure removal of androsta-1,4,6-triene-3,17 dione and any other radiochemical contaminants.

On the day of the assay, the frozen brains were dissected on a cold-plate by the method of Naftolin et al (1972), and the anterior hypothalamus containing the

median pre-optic area was removed into ice cold 0.05M phosphate buffer. Five or six hypothalami were pooled in each assay tube, and homogenized between teflon and glass. An NADPH regenerating system and additional buffer (pH=7) were added to make a total volume of 0.5 ml as described in section I, and the reaction was initiated by the addition of 10μCi of (1,2,6,7)-^{3}H-androstenedione (90 Ci/mmole). The reaction mixtures were incubated at 37° C for 3 hours, and the reaction terminated by the addition of 5 ml of methylene chloride. Samples were prepared for HPLC and subjected to chromatography on HPLC system A followed by chromatography on HPLC system B as described in section I (see Figure 10). Tritiated estrone was collected during chromatography on system B, and radioactivity was determined by liquid scintillation counting using a Packard Tri-Carb (Model 460 C). Estrone recovery was determined by U.V. absorbance, and the production of tritiated estrone per brain was calculated.

Statistical Analysis

Data were analyzed by a completely random analysis of variance (a=0.05), and differences between treatment groups in mean estrone production per brain were determined using Duncan's New Multiple Range Test (a=0.05).

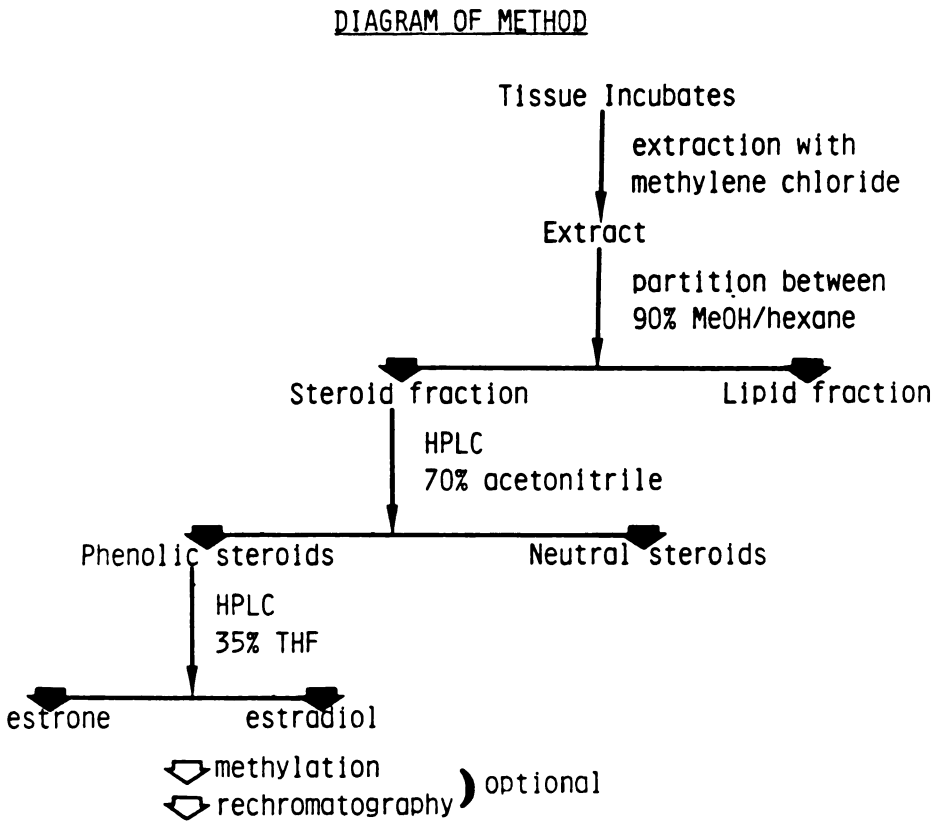

FIGURE 10. Summary of hypothalamic aromatase assay. Incubation conditions described in text.

RESULTS

Figure 11 summarizes the effects of sex, castration and PBB treatment on hypothalamic aromatase activity. Both control and PBB treated female brains had aromatase levels significantly lower than those displayed by any of the male brains (Table 3). PBB treated males exhibited a trend toward decreased aromatase, but the activity did not differ significantly from that seen in control males. PBB treatment also did not significantly affect aromatase in female brains. Castration on day 21 significantly decreased hypothalamic aromatase when compared to control male brains, although castration did not lower aromatase to the levels seen in female brains.

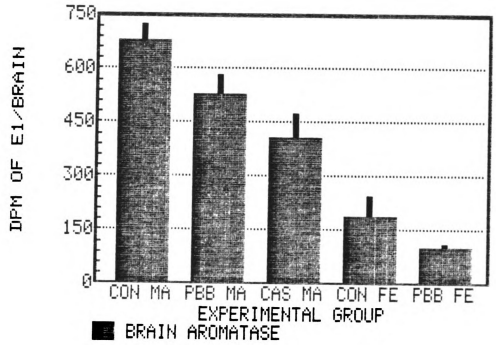

FIGURE 11. Effect of orchidectomy or PBB treatment at 21 days on the hypothalamic aromatase activity in adult male and female rats.

CON MA=control male, PBB MA=PBB treated males, CAS MA=castrate males, CON FE=control females, PBB FE=PBB treated females.

TABLE 3
Effect of orchidectomy or PBB treatment at 21 days on the hypothalamic aromatase activity in adult male and female rats.

group	brain aromatase DPM E_1 / brain
control male	676 ± 50 (3)[a]
PBB male	525 ± 57 (3)[a,b]
castrate male	407 ± 69 (3)[b]
control female	187 ± 53 (3)[c]
PBB female	100 ± 8 (3)[c]

expressed as mean ± s. e. m. (n)
a,b,c letters denote values which do not differ significantly from other values designated with the same letter.

DISCUSSION

These experiments demonstrate for the first time that pre-pubertal castration can affect hypothalamic aromatase activity in rats. A previous report by Reddy et al (1973) described increased aromatase in rabbits following adult castration. Since rabbits are reflex ovulators, hypothalamo-pituitary control of reproduction may be expected to differ substantially from that seen in rats or humans. The castration induced decrease in hypothalamic aromatase reported here may indicate that some parameters of central neuroendocrine differentiation are still plastic after the classical period of brain differentiation which ends at day 5 after birth (MacLusky and Naftolin, 1981).

Although castration at day 22 resulted in a significant decrease in aromatase activity, PBB treatment had no significant effect. The trend toward decreased aromatase seen in PBB treated male rats, although not statistically significant, was in the same direction as that seen in castrate animals, and may reflect the effect of decreased steroid levels expected with PBB. It must be kept in mind that these are preliminary experiments,

performed with few replicates (n=3). Future experiments with larger numbers of replicates should be more definitive.

Because of the effect on hypothalamic aromatase seen with castration, it is important to determine if perinatal PBB treatment will show the same effect; i.e. feminization of hypothalamic aromatase activity in the male brain. Because of the important role that aromatase plays in differentiation (McEwen et al 1980; Naftolin et al, 1975; Whalen and Olson, 1978; Clemens and Pomerantz, 1982), regulation of adult sexual behavior (Popolow and Ward, 1976; Whalen and Rezek, 1977), and regulation of gonadotropin secretion (Selmanoff et al, 1977; Naftolin et al, 1975; Worgul et al, 1981) it is critical to determine if aromatase activity is affected by reproductive toxicants such as PBB and phenobarbital (McCormack et al, 1979; Johnson et al, 1980; Gupta et al, 1982).

Perinatal PBB treatment may be expected to lower available steroid levels during brain differentiation, and thus impair the development of the hypothalamic aromatase system. Further experiments utilizing neonatal castration and PBB treatment will help determine the role, if any, that central effects play in the reproductive alterations seen following treatment by mixed-function oxidase inducers.

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

Exposure to PBBs results in induction of hepatic steroid metabolism. This may account for the reproductive effects associated with PBB exposure. In addition to direct effects on reproductive organs, decreases in circulating androgen titers may affect reproduction by altering central neuroendocrine control mechanisms. In addition, PBBs may exert central effects directly on the hypothalamus to alter aspects of reproductive physiology. In order to determine if PBB effects on reproduction involved the hypothalamus, a new, sensitive assay for hypothalamic aromatase was developed. Hypothalamic aromatase activity was measured in orchidectomized animals, and in control and PBB treated male and female rats. PBB induction of hepatic steroid metabolism was confirmed by _in vitro_ incubations of testosterone with hepatic microsomes.

The assay of hypothalamic aromatase employed dual HPLC chromatographies and liquid scintillation counting to isolate and quantify the estrone produced by aromatization of androstenedione by hypothalamic homogenates in vitro. This technique offered good recoveries of estrone (70 -

80%), and was sensitive enough to detect the production of less that 10 femtomoles of tritiated estrogen. The estrone isolated by the HPLC technique was of very high (greater than 95%) purity. Recoveries were calculated using U.V. absorbance of carrier estrogens added during incubation. The assay was a substantial improvement over existing methods, and was easily and rapidly performed.

Incubation of hepatic microsomes with testosterone has confirmed that a single dose of PBBs given at the time of weaning (22 day old rats), is sufficient to induce hepatic MFO activity in adulthood. PBBs increased overall testosterone metabolism in both male and female rats, whereas orchidectomy of 22 day old males decreased in vitro testosterone oxidation to levels comparable to those seen in female rats. Production of 7a-hydroxy-testosterone was increased in both male and female rats by PBB treatment, but 16a-hydroxytestosterone and 6B-hydroxy testosterone production was elevated only in male rats. Castration did not alter 7a-hydroxytestosterone production but did decrease the production of 16a- and 6B-hydroxy-testosterone to female levels.

Female rats had hypothalamic aromatase activities substantially below those seen in male animals. PBB treated males exhibited a trend toward decreased aromatase activity, but the activity did not differ significantly from that seen in control males. Castration on day 22 significantly decreased hypothalamic aromatase when

compared to control male brains, but did not lower aromatase levels to those seen in female brains.

These experiments demonstrated that hypothalamic aromatase may be affected by manipulation of the endocrine environment in weanling rats. Sexual differentiation of the hypothalamus is exquisitely sensitive to even small alterations in circulating steroid levels (Ward and Weisz, 1980), and endocrine manipulation during neonatal life may result in a deficit of hypothalamic development leading to permanent reproductive effects. PBBs may increase steroid metabolism during sexual differentiation, and thus produce lifelong defects in reproductive physiology. Further experiments are needed to determine whether the presence of PBBs during neonatal life is capable of permanently altering central neuroendocrine control of reproduction.

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LITERATURE CITED

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