STEROID METABOLISM IN GENETIC MUTANT MICE

METABOLISM OF TESTOSTERONE, PROGESTERONE AND ANDROSTENEDIONE IN THE LIVER, TESTES, HYPOTHALAMUS AND CORTEX OF NORMAL AND TESTICULAR FEMINIZED MICE

By

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ABSTRACT

The *in vitro* metabolism of testosterone, progesterone and androstenedione was studied in liver, testes, hypothalamus and cortex incubations of normal BALB/c ♂, BALB/c ♀ and a number of sex-mutant mice carrying the testicular feminization (*Tfm*) and sex-reversal (*sxr*) genes.

The metabolism of testosterone was studied in liver homogenates of BALB/c ♂, *Tfm* (o^+)♂ and *Tfm* (o^{hv})♂. Androstenedione was identified as the major metabolite of testosterone, in all incubations, indicating the presence of 17β-hydroxysteroid dehydrogenase activity. Comparison of the percentage conversions of testosterone to androstenedione suggested that of the three genotypes studied, the activity of 17β-hydroxysteroid dehydrogenase was lowest in the *Tfm* (o^{hv}) liver.

Similarly, the metabolism of testosterone and progesterone was studied by double-labelled tracer experiments using testes minces from BALB/c ♂, *Tfm* (o^+)♂ and *Tfm* (o^{hv})♂ mice. The conversion of progesterone to testosterone was greatest in BALB/c ♂ incubations and least in the *Tfm* (o^+) incubations. These data

suggest that the relative activities of the 17-ketoreductase for the conversion of androstenedione to testosterone are in the order: BALB/c ♂ > Tfm (o^{hv}) ♀ > Tfm (o^{+}).

Progesterone metabolism was studied in testis incubations of normal male (BALB/c ♂), testicular-feminized, sex-reversed (Tfm+(o^{hv})Blo/+++, sxr/+ ♂) and sex-reversed (+Ta++/+++, sxr/+ ♂) mice. Comparison of the amounts of androstenedione and testosterone formed from P suggests that differences are present in the relative activity of the testis 3-ketoreductase. The relative activity is in the order BALB/c ♂ > +Ta++/+++, sxr ♂ > Tfm+(o^{hv}) Blo/+++, sxr ♂ .

The metabolism of androstenedione was also studied in testes incubation of Tfm (o^{+})♀ and Tfm (o^{hv})♀ mice. The formation of testosterone was similar in all incubations. A number of unidentified metabolites of androstenedione were detected in the Tfm (o^{hv}) incubations which were not present in the Tfm (o^{+}) incubations.

Finally, the aromatization of testosterone was studied in hypothalamus and cortex incubations of normal

BALB/c ♂, BALB/c ♀, and testicular feminized Tfm (o^+)♂ and Tfm (o^{hv})♂ mice. A new, rapid method was developed for studying aromatization based on toluene-sodium hydroxide partitioning and a novel estrogen methylation procedure known as extractive alkylation. Using this method the formation of estrone and estradiol was detected. The conversion of testosterone to estradiol by hypothalamus minces was about 1.5 times greater in normal BALB/c ♂ than BALB/c ♀ and about the same as in Tfm (o^+) and Tfm (o^{hv}). Total aromatization by cortex minces was about 30-50% that of the hypothalamus. The major metabolite of testosterone in normal BALB/c ♂ and BALB/c ♀ incubations was estrone while in the Tfm (o^+) and Tfm (o^{hv}) incubations it was estradiol-17β.

The studies confirm the work of others which suggests that the metabolism of steroids is altered in the liver and testis of the Tfm (o^+)/Y♂ mouse. The present work extends these observations to include a number of other mice carrying the Tfm and sxr genes. Using the extractive alkylation technique aromatization was detected in Tfm hypothalamus and although the overall quantitative yields of estrogen from testosterone were similar to normals, qualitative differences were evident

which may be significant to the explanation of altered hypothalamic-pituitary gonadotrophin relationships in Tfm (o^{+}) and Tfm (o^{hv}) mice.

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ABBREVIATIONS AND TRIVIAL NAMES

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

testosterone (T)	4-androsten-17β-ol-3-one
progesterone (P)	4-pregnen-3,20-dione
androstenedione (A)	4-androsten-3,17-dione
dehydroepiandrosterone (DHA)	5-androsten-3β-ol-17-one
androstenediol ($AEOH_2$)	4-androsten-3α,17β-diol
5α-dihydrotestosterone (DHT)	5α-androstan-17β-ol-3-one
5α-androstanediol (AOH_2)	5α-androstan-3α,17β-diol
cholesterol	5-cholesten-3β-ol
pregnenolone	5-pregnen-3β-ol-20-one
androsterone	5α-androstan-3α-ol-17-one
3,17β-dimethoxyestriol	3,17β-dimethoxy-1,3,5(10)-estratrien-17β-ol
2,3-dimethoxyestrone (2,3-di0MeE_1)	2,3-dimethoxy-1,3,5(10)-estratrien-17-one
estradiol-17α($E_2$17α)	1,3,5(10)-estratrien-3,17α-diol
estradiol-17β($E_2$17β)	1,3,5(10)-estratrien-3,17β-diol
estriol (E_3)	1,3,5(10)-estratrien-3,16α,17β-triol
estrone (E_1)	3-hydroxy-1,3,5(10)-estratrien-17-one
2-hydroxyestrone (2-0HE_1)	2,3-dihydroxy-1,3,5(10)-estratrien-17-one
3-methoxyestradiol-17α ($E_2$0Me-17α)	3-methoxy-1,3,5(10)-estratrien-17α-ol

3-methoxyestradiol-17β (E_2OMe-17β)	3-methoxy-1,3,5(10)-estratrien-17β-ol
3-methoxyestriol (E_3OMe)	3-methoxy-1,3,5(10)-estratrien-16α-17β-diol
2-methoxyestrone (2-OMeE_1)	2-methoxy-3-hydroxy-1,3,5(10)-estratrien-17-one
3-methoxyestrone (E_1OMe)	3-methoxy-1,3,5(10)-estratrien-17-one
2-hydroxy-3-methoxyestrone (2-OHE_1OMe)	3-methoxy-2-hydroxy-1,3,5(10)-estratrien-17-one
Tfm	testicular feminization locus on mouse X-chromosome
Ps	pseudohermaphrodite
MIH	Mullerian-inhibiting hormone
LH	luteinizing hormone
FSH	follicle-stimulating hormone
TMS	trimethylsilyl
THAH	tetrahexylammonium hydroxide
PMA	phosphomolybdic acid
PPO	2,5-diphenyloxazole
POPOP	1,4-bis-[-2(5-phenyloxazolyl)] benzene
N_2	nitrogen
CH_3I	methyl iodide
CH_2Cl_2	methylene chloride
MeOH	methanol
NaOH	sodium hydroxide

G-6-P	glucose-6-phosphate
G-6-PD	glucose-6-phosphate dehydrogenase
NADP	nicotinamide adenine dinucleotide phosphate monosodium salt
NADPH	nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt
PC	paper chromatography
GC	gas chromatography
MS	mass spectroscopy
EA	extractive alkylation
UV	ultraviolet
FID	flame ionization detector
mm	millimeter
cm	centimeter
I.D.	inner diameter
wt	weight
ml	milliliter
mg	milligram
µg	microgram
hr	hour
min	minute
^{o}C	degrees Centigrade
cpm	counts per minute
dpm	decompositions per minute

rpm	revolutions per minute
µamp	microampere
µCi	microCurie
eV	electron volts
N	normality
M	molarity
S.E.	standard error from the mean
R_t	the retention time of the substance under consideration in a chromatographic system measured from the time of injection to detection.
R_f	the ratio of the velocity of the substance under consideration to the velocity of the mobile phase in a chromatographic system.

INTRODUCTION

Definition

"Testicular feminization" was first used by Morris (1953) to describe a clinically recognizable syndrome in man characterised by: (1) female habitus with well-developed breasts; (2) absent or scanty axillary, pubic and facial hair; (3) female external genitalia with the vagina ending blindly; (4) absence of female internal genitalia; (5) inguinal or intra-abdominal testes; (6) detectable production of estrogens and androgens; (7) elevated pituitary gonadotropins. The term is in part, a misnomer, since the testes are not "feminized" containing no follicular derivatives (Morris, 1963). Nevertheless, it is in accepted use to denote an inherited disorder of sexual differentiation recognized in man, mouse (Lyon, 1970), rat, (Stanley and Gumbreck, 1964) and cattle (Short, 1967).

Two distinct forms of the syndrome have been delineated. In the "complete" form there is a striking resistance to the effects of endogenous and exogenous androgens. (Table 1). The clitoris is small and normal (Morris, 1963; Lyon and Hawkes, 1970; Bardin et. al., 1973). In the "incomplete" form, some responses to androgens are present and the clitoris is enlarged and penis-like (Morris, 1963; Himathongkam et. al., 1974; Ohno et. al., 1973; Bardin et. al., 1973).

Etiology

The etiology of testicular feminization has been

unclear until recently. It is the prime example of phenotypic female development in the presence of a genetic male (XY) genotype. Females are carriers but the exact mechanism of genetic transmittance has not been determined except in the mouse, where X-linkage has been established (Lyon and Hawkes, 1970).

In accordance with the theories formulated by Wolff (1953) and Jost (Jost, 1953, 1970; Jost et. al., 1973), differentiation of the male internal and external genitalia is mediated by androgen secreted by the fetal testes. Hauser (1963) first proposed that "testicular feminization is the inevitable consequence of early intrauterine testicular insufficiency". This proposal has been difficult to test in the human since early clinical recognition of the syndrome is a rare occurrence (Money and Ehrhardt, 1972). However, in the testicular feminized (Tfm)[1] mouse, early testicular insufficiency has been disproved by Goldstein and Wilson (1972). Testosterone (T) synthesis in newborn Tfm mice is normal, but progressively declines with age, until in the adult, androgen synthesis

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[1]Abbreviations for the testicular feminization characteristic vary according to authors and species under consideration. The notation used here for the mouse is the generally used one of Ohno et. al. (1973). In this case the Tfm is thought to represent an actual gene product, although its exact nature is unknown at present. The linkage map for the mouse X-chromosome carrying Tfm is described in Figure 1.

occurs at very low, but detectable levels. Furthermore, androgen administration to pregnant mice carrying the Tfm fetus does not result in any virilization of the Tfm offspring. This direct evidence supports the view that the failure of male sexual differentiation is due to androgen resistance during embryogenesis. In utero, deficient androgen effect is indicated by female urogenital sinus development with failure of Wolffian duct system development. The male development of these structures is androgen-dependent and they will be female in the absence of androgens (Jost, 1953). As androgens do not inhibit the development of the Mullerian system, the absence of Mullerian derivatives has been explained by the presence of a testes and the production of the "Mullerian-inhibiting hormone" (MIH) recently demonstrated to be of a protein nature (Josso, 1972) and to be secreted by Sertoli cells (Josso, 1973). MIH inhibits development of the uterus and upper two-thirds of the vagina.

A wide variety of explanations have been offered to explain the occurrence of testicular feminization. Maternal interference with the "testicular inductor substance" whatever that substance might be was proposed by Witschi et. al. (1957). Decreased cellular uptake of T due to an increased capacity of the T-binding globulin has been suggested as a possible cause (Tremblay et. al., 1972).

All other proposals on etiology, can be classified and divided according to two theories; the "receptor theory" and the "metabolism theory". According to the former, as first proposed by Wilkins (1957), there is an inherited insensitivity of target organs to androgens. The metabolism theory presumes the existence of metabolic defect(s) leading to reduced (or increased) formation of steroids. Regardless of the mechanism of testicular feminization, the lack of effect of steroids on a number of steroid-dependent responses has been well demonstrated (Table 1).

Consistent with the concept that the actions of androgen are mediated in target cells through binding with specific cytosol receptors, decreased binding would be expected in androgen target cells in testicular feminization (Liao, 1974; King and Mainwaring, 1974). In the Tfm mouse and pseudohermaphrodite (Ps) rat deficient cytosol androgen binding has indeed been demonstrated by a wide variety of methods (Table 2).

The availability of a number of genetic mutant mice carrying the Tfm gene (Lyon and Hawkes, 1970; Ohno, 1971; Ohno et. al., 1973) prompted the present study of steroid metabolism in various tissues of the Tfm mouse. According to the "steroid metabolism" theory of testicular feminization, steroid metabolism in some tissues from mice bearing the Tfm gene and other modifying genes affecting sexual differentiation (see Fig.1) should be different from genetically normal male mice. Testes, liver and hypothalamus were chosen for study.

FIGURE 1: Genetics of Tfm in the mouse.

1a: genotypes.

1b: origin of Tfm (o^{hv})

(from Ohno et. al., 1973)

Tfm testicular feminization locus

Ta tabby coat color locus

(o) Cattanach's "controlling element" locus (Cattanach et. al., 1970)

Blo blotchy coat color locus

+ wild type allelle

sxr sex reversal (autosomal) locus (Cattanach et. al., 1971)

$\equiv$ equivalent to

GENETICS OF T_{fm} IN THE MOUSE

Genotypes

$T_{fm}+(O^{\dagger})+/Y\ \male \equiv T_{fm}+++/Y\ (XY)$

$T_{fm}+(O^{hv})\ Blo/Y\ \male \equiv T_{fm}+O^{hv}\ Blo/Y\ (XY)$

$+\ Ta++/+++,\ sxr\ \female \equiv +\ Ta++/X\ (XX)+(sxr/+)$

$T_{fm}+(O^{hv})\ Blo/+++,\ sxr\ \female \equiv T_{fm}+O^{hv}\ Blo/X\ (XX)+(sxr/+)$

FIGURE 1a

ORIGIN OF $T_{fm}(O^{hv})$ ..... from Ohno et al., 1973

$T_{fm}+(O^{hv})\ Blo/+++\ \male$ (extreme variegation of Blo)

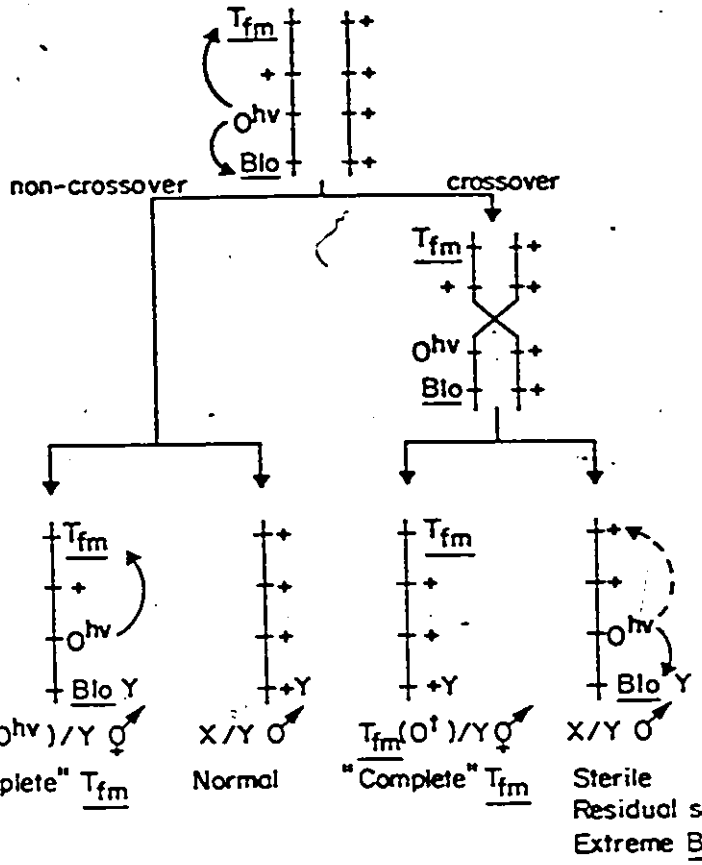


FIGURE 1b

Table 1. Hormonal responses in testicular feminization

1. Rat

Administered	Expected response	Observed response	Reference
T or DHT	(+) liver ethylmorphine demethylase (+) Δ^4-steroid reductase	no effect	Bullock et.al.(1971)
Tenthanate	(-) LH, FSH (-) preputial gland wt. (+) Leydig cell size (+) 3β HSDH (+) pituitary wt.	(+) LH, FSH, preputial gland wt. no effect on rest	Sherins et.al.(1971) Sherins and Bardin (1971)
E.benzoate	(-) LH, FSH (+) pituitary wt. (-) Leydig cell size (-) 3β HSDH	(-) LH,FSH (+) pituitary wt. (-) Leydig cell size (-) 3β HSDH	Sherins et.al.(1971) Sherins and Bardin (1971)
Progesterone	pituitary wt. preputial gland wt. no effect	no effect	Sherins et.al.(1971) Sherins and Bardin (1971)
T	(+) testis wt.	(-) testis wt.	Stanley et.al.(1973)
T	(+) Urinary 2μ-globulin	no effect	Neuhaus and Irwin (1972)
T or DHT	(+) hepatic hexobarbital metabolism (+) preputial gland wt. (+) adrenal gland wt.	no effect	Bardin et.al.(1970)
T or DHT	(+) renal-l-gulonolactonase	no effect	Grossman et.al.(1970
T or DHT	(+) preputial gland DNA, RNA, protein synthesis	no effect	Sherins and Bardin (1971)
T or DHT	(+) citrate excretion nitrogen retention	no effect	Chan and Allison (1969)

Table 1 (cont.)

2. Human

Administered	Expected response	Observed response	Reference
LH-RF	(+) LH	(+) LH	Zârate et.al. (1975)
estradiol	(-) LH, FSH	(-) LH, FSH	Zârate et.al.(1975)
Clomiphene	(+) LH, FSH	no effect	Zârate et.al.(1975)
methyl T	(+) clitoral size (+) pubic hair (-) vocal tone	no effect	Wilkins (1957)
T	(+) nitrogen retention	no effect	Bahner and Schwarz (1962)
E	(+) TeBG	(±) TeBG	Mauvais-Jarvis (1972) Tremblay(1972

3. Mouse

Administered	Genotype	Expected response	Observed response	reference
DHT	Tfm (o^+)/X ♀ (pregnant)	in utero virilization of Tfm (o^+)/Y ♂ offspring	no effect	Goldstein and Wilson(1972)
T	Tfm (o^+)/Y ♂ Tfm (ohv)/Y ♂	(-) LH (-) LH	no effect (-) LH	Kan et.al.(1974)
DHT	Tfm (o^+)/Y ♂	(+) kidney AdH (+) -glucuronidase		
T	Tfm (o^+)/Y ♂	(+) pituitary castration cells	no effect (+) pituitary castration cells	Dofuku et.al.(1971) (1973) Itakura and Ohno
T	Tfm (o^+)/Y ♂ Tfm /+ ♀	(+) submaxillary gland NGF	no effect (±)submaxillary gland NGF	Lyon et.al.(1973)

Table 1 (cont.)

Administered	Genotype	Expected response	Observed response	reference
E benzoate	Tfm (o^+)/Y ♂	(+) testicular lipids	no effect	Chung and Hamilton (1975)

T testosterone
E estrogen
DHT 5α-dihydrotestosterone
LH-RF lutenizinghormone-releasing factor
(+) increased
(-) decreased
(±) small increase or decrease

Table 2. "Steroid receptors" in testicular feminization

1. Mouse

tissue	"receptor"	specificity	"binding" relative to normal XY	reference
kidney	cytosol	androgen	(-)	Attardi and Ohno (1974) Bardin and Bullock (1974) Gehring et.al. (1971)
kidney	cytosol	estrogen	no change	Bullock and Bardin (1975)
kidney	nuclear	androgen	no change	Drews et.al.(1972)
submandibular gland	cytosol	androgen	(+)	Wilson and Goldstein (1972)
submaxillary gland	cytosol	androgen	no change	Lyon et.al. (1973)
hypothalamus	cytosol	androgen	(-)	Fox (1975)
hypothalamus	cytosol	estrogen	no change	Fox (1975)

2. Rat

tissue	"receptor"	specificity	binding relative to normal XY	reference
preputial gland	cytosol	androgen	(-)	Bullock and Bardin (1972) Bardin and Bullock (1974)

There is some evidence for resistance to T or 5α-dihydrotestosterone (DHT) action on the testes (Stanley et al., 1973, liver (Bullock et. al., 1971), kidney (Dofuku et. al., 1971) and hypothalamus (Kan et. al., 1974). Deficient androgen receptor activity has been reported in the testicular feminized liver (Milin and Roy, 1973), kidney (Attardi and Ohno, 1974; Bardin and Bullock, 1974) and hypothalamus (Fox, 1975). The relationship between steroid metabolism and alterations in steroid receptor activity in these tissues is not known.

Steroid metabolism

The metabolic pathways for the production of progestogens, androgens and estrogens are shown in Fig. 2. Enzymes for the synthesis of cholesterol from acetate are in the microsomes as are those forthe conversion of pregnenolone to T, while the enzymes required for side-chain cleavage of cholesterol are in the mitochondria.

Testes

Interstitial tissue is the principal source of testicular androgens, although seminiferous tubules are also capable of androgen biosynthesis from pregnenolone and progesterone (P) but not from cholesterol (Christenson and Mason, 1965; Hall et. al., 1969). The formation of C_{21}-17-hydroxysteroids and C_{19}-steroids from pregnenolone andprogesterone has also been demonstrated in germ cells from immature rat testes

(Yamada et. al., 1973). The production and secretion of estrogens by the mammalian testes has long been established (Beall, 1940), although the cellular source(s) have not been fully established. Dorrington and Armstrong (1975) have recently demonstrated specific estrogen synthesis from T in cultures of rat Sertoli cells.

Decreased production of testicular androgens was the first suspected abnormality of steroid metabolism in testicular feminization (Hauser, 1963). Peripheral inactivation of T by increased conjugation or aromatization of the A-ring has also been proposed (Morris, 1963). Some of the known or suspected defects of steroid metabolism in testicular feminization include (1) decreased 3β-hydroxysteroid dehydrogenase-isomerase (Morris, 1963; Bell, 1975), (2) decreased 17β-hydroxysteroid dehydrogenase, decreased 17α-hydroxylase (Bardin et. al., 1973), (3) decreased 5α-reductase (Mauvais-Jarvis, et. al., 1968; Mauvais-Jarvis et.al., 1970), (4) increased 20-keto-reductase (Bardin, et. al., 1973).

Collectively, the steroid metabolism studies of human testes are, at best, inconclusive. Methodological differences and the lack of control tissue make comparison of the relative steroid metabolizing activities of testicular feminized verus normal testes difficult. However, many investigators have shown that the testicular feminized testes is capable of synthesizing androgens and estrogens (Table 3)

FIGURE 2: Metabolic pathways for the production of progestogens, androgens and estrogens

17αOHase	17α-hydroxylase
17-20-lyase	C_{17-20}-lyase
17βOHSDH	17β-hydroxysteroid dehydrogenase
3βOHSDH	3β-hydroxysteroid dehydrogenase
isomerase	Δ^4-Δ^5 steroid isomerase
5α-reductase	
3-ketoreductase	
19-OHase	C_{19}-hydroxylase
19-OHDH	19-hydroxysteroid dehydrogenase
10-19 lyase	C_{10-19}-lyase
17βHSDH	17β-hydroxysteroid dehydrogenase (estrogen specific)

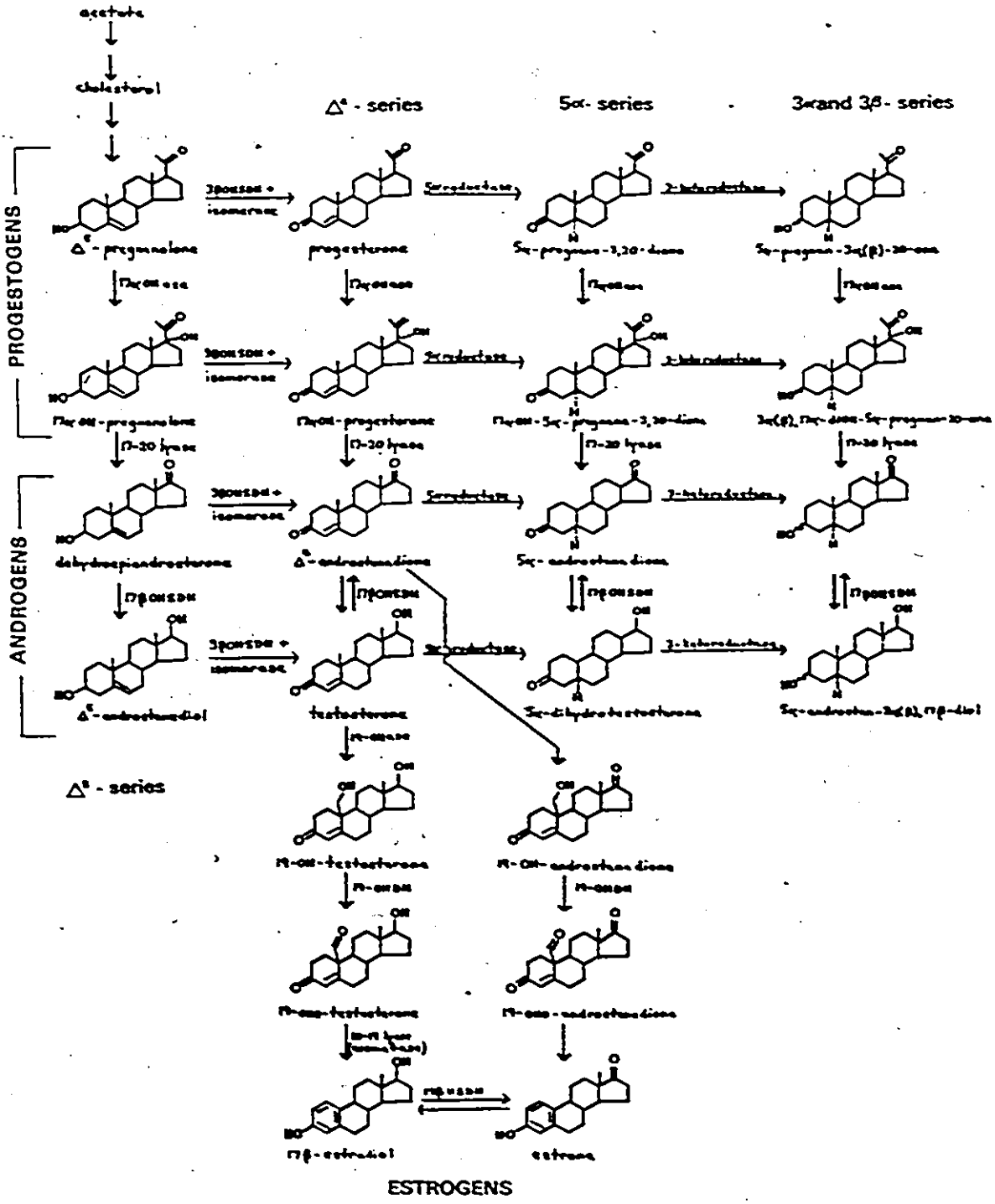

acetate
cholesterol
Δ⁴ - series
5α- series
3α and 3β - series
PROGESTOGENS
ANDROGENS
Δ⁴ - pregnenolone
progesterone
5α - pregnane - 3,20 - dione
5α - pregnane - 3α(β) - 20 - one
17α OH - pregnenolone
17α OH - progesterone
17α OH - 5α - pregnane - 3,20 - dione
17-20 lyase
dehydroepiandrosterone
Δ⁴ - androstenedione
5α - androstane dione
Δ⁵ - androstenediol
testosterone
5α - dihydrotestosterone
Δ⁵ - series
17 - OH - testosterone
17 - OH - androstenedione
17 - oxo - testosterone
17 - oxo - androstenedione
17β - estradiol
estrone
ESTROGENS

Figure 2

Table 3. Steroid metabolism by tissues in testicular feminization

TESTES - HUMAN

incubated	recovered: neutral	recovered: phenolic	reference
^{14}C-progesterone ^{14}C-androstenedione ^{14}C-testosterone	testosterone, androstenedione testosterone androstenedione	no estrogen	Griffiths (1963)
^{14}C-progesterone	testosterone, androstenedione 20α- hydroxypregn-4-ene-3-one 17α,20β-dihydroxypregn-4-en-3-one 16α-hydroxyprogesterone	no estrogens	"
^{14}C-17α -hydroxy-progesterone	no metabolism		"
^{14}C-androstenedione	testosterone	unidentified phenol with mobility between E_1 and E_2	"
^{3}H-pregnenolone	17α-hydroxypregnenolone dehydroepiandrosterone androstenedione testosterone	E_1 (.05%) E_2 (0.06%)	Morris and Mahesh, (1963)
^{3}H-dehydroepiandro-sterone	androstenedione testosterone	E_1 (.25%) E_2 (.3%)	"
^{14}C-progesterone	17α-hydroxyprogesterone androstenedione	E_1 (.08%) E_2 (.08%)	"

Table 3 (cont.)

incubated	recovered: neutral	recovered: phenolic	reference
^{14}C-androstenedione	testosterone	no E_1, E_2 "phenolic substance with mobility between E_1 and E_2"	Pion et.al.(1965)
^{14}C-dehydroepiandrosterone	dehydroepiandrosterone sulphate	no estrogens	"
^{14}C-progesterone	androstenedione 20α-hydroxypregnen-3-one, 17α-hydroxyprogesterone testosterone	no estrogens	Charreau and Villee (1968)
^{14}C-pregenolone	17α-hydroxypregnenolone dehydroepiandrosterone androst-5-ene-3,17-diol	no estrogens	"
^{3}H-dehydroepiandrosterone + ^{14}C-androstenedione	testosterone, androst-5-ene-3,17-diol	"equilenin-like" material	"
^{14}C-testosterone	androstenedione	no estrogens	Wade et.al.(1968)
^{14}C-androstenedione	testosterone 19-hydroxytestosterone	no estrogens	
^{14}C-estrone		17β-estradiol 2-methoxyestradiol 2-methoxyestrone other unidentified products	

Table 3 (cont.)

2. TESTES - RAT

incubated	recovered	reference
testosterone	5α-dihydrostestosterone 5α-androsten-3α,17β-diol	Bullock and Bardin (1973)
progesterone	17α-hydroxyprogesterone, androstenedione, testosterone androstanediol, androsterone	Coffey et.al. (1972)
testosterone	androstenedione, 5α-androsten-3α,17β-diol, androsterone	"
androstenedione	testosterone, 5α-androsten-3α,17β-diol, androsterone	"
pregnenolone	progesterone, 17α-hydroxy-pregnenolone, androstenedione, testosterone	Schneider and Bardin (1970)
17α-hydroxyprogesterone	androstenedione, testosterone	"
androstenedione	testosterone	"
estrone	17β-estradiol	"

Table 3 (cont.)

incubated	recovered: neutral	recovered: phenolic	reference
^{14}C-progesterone	androstenedione, 17α-hydroxyprogesterone, 16α-hydroxyprogesterone	no estrogens	Sharma et.al (1965)
^{14}C-androstenedione	testosterone, 6β-hydroxytestosterone	estrone, 17β-estradiol, possibly equilenin	"
^{14}C-testosterone	androstenedione, 6β-hydroxytestosterone	estrone, 17β-estradiol	"
^{14}C-17α-hydroxyprogesterone	testosterone, 17,20β-dihydroxypregn-4-ene-3-one	no estrogens	French et.al. (1967)
^{14}C-pregnenolone	17α-hydroxypregnenolone, pregn-3β,17α,20α-triol dehydroepiandrosterone progesterone, 17α-hydroxyprogesterone, testosterone	no estrogens	Neher, et.al. (1965)
^{14}C-17 -hydroxypregnenolone	pregn-5-ene-3β,17α,20α-triol dehydroepiandrosterone, progesterone, 17α-hydroxyprogesterone, testosterone	no estrogens	"
^{14}C-progesterone	pregn-4-one-20α-ol-3-one 17α-hydroxyprogesterone, 16α-hydroxyprogesterone, testosterone	no estrogens	"
^{14}C-androstenedione	testosterone	estradiol (0.5%) estrone (0.2%)	"

Table 3 (cont.)

incubated	recovered	reference
testosterone	5α-dihydrotestosterone, 5α-androsten-3α,17β-diol	Bardin et.al. (1973)
testosterone	5α-dihydrotestosterone, 5α-androsten-3α,17β-diol	Bullock and Bardin (1973)
3. TESTES - MOUSE		
androstenedione	testosterone	Blackburn et.al. (1973)
progesterone	testosterone	Goldstein and Wilson (1972)
pregnenolone	testosterone	"
17α-hydroxyprogesterone	testosterone	"
androstenedione	testosterone	"
testosterone	5α-dihydrotestosterone	"

and any differences from normal testes therefore would appear to be relative rather than absolute. Defects in steroid metabolism have been suggested as the main abnormality in testicular feminization in the human. Consistent with the view that DHT is the active androgen in many tissues, Mauvais-Jarvis proposed that 5α-reductase deficiency was the cause of testicular feminization (Mauvais-Jarvis et al., 1968), although according to Goldstein and Wilson (1972) and Karazia et. al., (1969) 5α-reductase activity is normal.

Production of T in the human testes occurs predominantly by the Δ^5-pathway (Bell and Lacy, 1974). Bell (1975) has demonstrated <u>in vitro</u> T production by the Δ^5-pathway in a number of cases of "complete" and "incomplete" testicular feminization. An abnormal accumulation of androstenediol ($AEOH_2$) and dehydroepiandrosterone (DHA) from T, <u>in vitro</u> has been demonstrated in human testes, in both the "complete" and "incomplete" forms (Bell and Lacy, 1974). According to Bell (1975) the major biochemical difference between the "complete" and "incomplete" forms of the syndrome is the greater production and accumulation of C_{21} and C_{19}-Δ^4 steroids from the C_{21} and C_{19}-Δ^5 steroid precursors in the "incomplete" than in the "complete" form, indicating the relatively greater activity of the C_{21}-3β-hydroxysteroid dehydrogenase-isomerase enzyme in the "incomplete" form.

Production of T in the mouse testes, on the other

hand, occurs predominantly by the Δ^4-pathway (Tsujimura and Matsumoto, 1974; Tsujimura et. al., 1975).

The availability of control tissues has made the study of steroid metabolism in the Tfm mouse and Ps rat useful. In the Tfm mouse, Blackburn et. al. (1973) found that testicular minces metabolized 2-7 times less androstenedione (A) to T than normal male littermates. Schneider and Bardin (1970) and Vanha-Perttula et. al. (1970) have demonstrated that the Ps rat testes differs from normal in the overall pattern of steroid metabolism. Testes converted Δ^5-pregnenolone to a number of metabolites which accumulated to a greater extent in incubations of Ps tissues than controls. Increased prepubertal formation of the reduced T metabolites androsterone and 5α-androstanediol (AOH_2) which does not decrease postpubertally in Ps rats, as it does in normals, has been demonstrated by Coffey et. al. (1972). Studies of the testicular feminized rodent testes collectively suggest that (1) androgen biosynthesis in newborns is normal, (2) decreased 17β-hydroxysteroid dehydrogenase and possibly other enzyme abnormalities are evident post-pubertally (Bardin et. al., 1973).

Hypothalamus

Ohno and colleagues have described the production of a variant Tfm line, exhibiting the development of some male characteristics, as a result of a change in the nearby "controlling element" (Cattanach, 1970), which modifies the expression of the

Tfm mutation. In their stock (Ohno, et. al., 1973), mice of genotypes Tfm + (o^+) $+/\sigma^{\prime}$ [Tfm(o^+)] and Tfm + (o^{hv}) Blo $/\sigma^{\prime}$ [Tfm(o^{hv})] (Fig. 1) resemble development in the "complete" and "incomplete" forms respectively of testicular feminization in the human. Thus, Tfm (o^{hv}) mice show no vaginal opening at puberty, the presence of an enlarged penis-like clitoris with corpus spongiosium and a small epididymis and vas deferens (Ohno et. al., 1973).

Kan et. al. (1974) have shown that in Tfm (o^+) mice, luteinizing hormone (LH) levels are higher than normal. Estrogen but not T suppresses high post-castration LH and FSH levels. In Tfm (o^{hv}) mice, LH levels are normal and T or estradiol-17β (E_2) are both capable of suppressing high post-castration LH and FSH levels. A mechanism for the restoration of T sensitivity to the Tfm (o^{hv}) hypothalamus could involve hypothalamic steroid receptors (Fox, 1975), or the formation of estrogens in the hypothalamus (Naftolin et. al., 1975).

The ability of the mammalian brain to aromatize androgens was first suggested by Knapstein et. al., (1968) and has been corroborated by various workers in a number of preparations (Flores et. al., 1973a; Flores et. al., 1973b; Naftolin et. al., 1971a; Naftolin et. al., 1971b; Naftolin et.al., 1972; Weisz and Gibbs, 1974; Weisz and Philpott, 1971; Lieberburg and McEwen, 1975). However, the function of the estrogens formed in the brain remains unclear. According to

Naftolin and Ryan (1975), neonatal exposure to T induces aromatizing enzymes in the hypothalamus responsible for the conversion of androgens to estrogens.

If androgens are further available as substrate, hypothalamic aromatization by the induced enzyme occurs, resulting in a high intracellular concentration of estrogen. A similar requirement for T stimulation of Sertoli cell aromatization enzymes has recently been proposed by Dorrington and Armstrong (1975). Naftolin et. al. (1975) further suggest that estrogen formed from androgens could mediate the androgenic effects pertaining to sexual differentiation of the brain and regulation of gonadotrophic secretions.

Some evidence for the possible involvement of aromatization in the control of gonadotrophin levels comes from Faiman and Winter (1974) in their study of the complete form of testicular feminization in the human. They found that T but not DHT was capable of suppressing LH and FSH levels. T but not DHT can be converted to 17β-estradiol (E_2-17β). This suggests that, if aromatization is important in hypothalamic control of gonadotrophins, as proposed by Naftolin (Naftolin et. al., 1975) then a defect in testicular feminization may involve the inability of the hypothalamus to aromatize T. By the same argument, according to the work of Kan et. al. (1974) the presence of the modifying gene in Tfm (o^{hv}) mice should also affect aromatization. To test this hypothesis,

aromatization was investigated in normal male, female, Tfm (o^+) and Tfm (o^{hv}) hypothalamus and cortex.

Liver

Attardi and Ohno (1974) were not able to demonstrate hepatic cytosol androgen receptors in the liver of normal or testicular feminized mice. However, cytosol binding of DHT in the Ps rat is deficient (Milan and Roy, 1973). Gustafsson et. al. (1975) have demonstrated that neonatal "imprinting" of hepatic steroid metabolism is sex-dependent and under hypothalamo-hypophyseal and testicular androgen control. Only one brief report concerning the metabolism of steroids in the liver during testicular feminization has appeared. Bullock et. al. (1971), found that the hepatic reduction of T in crude liver homogenates was greater in Ps rats than in normal males. However, species differences exist between the normal rat and mouse in the hepatic metabolism of T, with little ring-A reduced metabolites being produced in the mouse (Jagarinec et. al., 1967). To test the hypothesis that neonatal androgen insensitivity may irreversibly (Gustafsson and Stenberg, 1974a,b) alter adult Tfm hepatic steroid metabolism, the metabolism of T was studied in liver homogenates of normal male mice and compared with Tfm (o^+) and Tfm (o^{hv}) liver.

Objectives of the present study

The specific objectives of this work were as follows:

(1) To compare some aspects of the metabolism of testosterone, progesterone and androstenedione in the liver and testes of a number of genetic mutant mice carrying the Tfm and sxr genes.

(2) To study in vitro aromatization of testosterone in the normal and Tfm mouse hypothalamus and cortex. Problems relating to the measurement of estrogen were approached using a new method for the preparation of estrogen methyl ethers.

GENERAL METHODS

(a) Measurement of Radioactivity

Aliquots of samples to be counted were evaporated under a stream of dry nitrogen or air with warming, in 5 dram vials and dissolved in 10 ml toluene containing 0.5% (w/v) PPO and 0.1% POPOP. Compounds insoluble in toluene were dissolved in 0.1 - 0.5 ml methanol prior to addition of the scintillation fluid. Radioactivity in aqueous solutions was measured by dissolving a 2 ml aliquot in 14 ml of a cocktail containing 0.55% (w/v) PPO and 0.017% (w/v) POPOP in toluene: Triton-X (2:1). All samples were counted for a time sufficient to give a standard deviation of 2% or better, except for samples containing less than 200 dpm which were counted for 50 minutes each.

All liquid scintillation counting was performed using the Beckmann LS-233 System. Two instruments of the same model were used. Due to small differences in the counting efficiencies of each instrument, separate quench calibration curves were constructed for each instrument using a series of quenched standards.

Single label counting was accomplished using a full ^{3}H- or ^{14}C- channel at a gain setting of 260. At this setting, the efficiencies of counting ^{3}H

and ^{14}C were about 48% and 75% in unquenched samples and about 35% and 60% in samples quenched with methanol. The values obtained for cpm in each sample were corrected for quenching using the standard quench curve to obtain a value in dpm for each sample.

Samples containing both ^{3}H and ^{14}C to be counted simultaneously were counted using the half-^{3}H channel and the full ^{14}C-channel at a gain setting of 305. To total ^{3}H and ^{14}C counts were calculated using the discriminator ratio method as described by Ulick (1961). The equations used were as follows:

$$\text{total } {}^{3}H = N_1 - \frac{N_2}{b}$$

$$\text{and total } {}^{14}C = N_2 - N_1 a$$

where N_1 = total counts in ^{3}H-channel

N_2 = total counts in ^{14}C-channel

$$a = \frac{{}^{3}H \text{ in } {}^{14}C\text{-channel}}{{}^{3}H \text{ in } {}^{3}H\text{-channel}}$$

$$b = \frac{{}^{14}C \text{ in } {}^{14}C\text{-channel}}{{}^{14}C \text{ in } {}^{3}H\text{-channel}}$$

Appropriate standards containing ^{3}H and ^{14}C were counted with each set of vials to determine the "a" and "b" ratios. In most cases, the value of "a" was small enough to be disregarded, while the value of "b" was 6.0 - 7.2. After correction for spill into each channel, all counts were converted to dpm.

(b) Chromatography

The compositions of the solvent systems used for chromatography are given in Table 4.

1. Paper Chromatography (PC)

All PC was carried out as according to Bush (1952), at room temperature (20-22°C). The upper and lower phases were occasionally replenished to make up for losses due to evaporation. Whatman # 1 chromatography paper (46 x 58 cm) was cut lengthwise into 3 cm strips with 10 cm top margins, 2 cm bottom margins and used for all paper chromatography separations without prior washing. Transfers to the starting (10 cm) line were made in small volumes (10 µl) of chloroform:methanol (1:1) or methanol under a stream of cold air from a hair dryer. The paper was hung overnight in a chromatography tank to come to equilibrium and developed with 40 ml upper phase the following day. The solvent was allowed to advance 45 cm from the origin. Strips were cut and scanned for radioactivity in a Nuclear-Chicago Actigraph III paper strip scanner in the case of radioactive material separations or, in the case of standards, visualized under U.V. light or sprayed with 3.5% phosphomolybic acid (PMA) and heated at 80-100°C for 2-5 min. Nonradioactive reference standards were included with each chromatography run, since

large variations in R_f values were observed between different runs. Radioactivity was eluted from strips cut from papers after chromatography in a descending system consisting of a 10 ml glass syringe fitted with a 18 gauge Luer-Lock needle on which paper strips were hung and eluted with 10 ml methanol. This procedure was found to remove 95-100% of the radioactivity on test strips spotted with known amounts of radioactive standards. The eluates from paper strips were collected in 15 ml conical centrifuge tubes and dried at 40-50°C under a constant stream of air previously passed through Drierite to remove moisture, or alternatively, the eluates were dried under a constant stream of nitrogen (N_2) with warming on a constant temperature sand bath. The eluates were covered and stored in the cold room (approx. 4°C) until further work-up.

2. Thin-Layer Chromatography (TLC)

TLC was performed using 20 x 20 cm sheets of silica gel F-254 (0.25 mm thickness) on plastic backing and supported on glass plates. Spotting of extracts or standards was done in chloroform: methanol (1:1) or methanol respectively under a stream of cold air. All TLC separations were done at room temperature with the exception of TLC-B (Table 4), where better separation was obtained at 4°C. Standards were included with each run, and were located using U.V. light or PMA with heating. ^{14}C-labelled steroids were located by

Table 4. Composition of chromatography systems

paper chromatography	composition (parts by volume)
Bush A	heptane:methanol:water (5:4:1)
Bush B_3	heptane:benzene:methanol:water (33:17:40:10)
Tol/PE	toluene:light petroleum, methanol: water (5:5:8:2)
thin-layer	
TLC-A	chloroform:methanol (1:1)
TLC-B	chloroform:methanol (49:1)
TLC-C	benzene:ethyl acetate (2:1)
TLC-D	benzene:ethyl acetate (13:1)
TLC-E	cyclohexane:ethyl acetate:ethanol (9:9:2)
TLC-F	ethyl acetate: n-hexane:ethanol (16:3:1)
TLC-G	chloroform:ethanol (9:1)

autoradiography using Kodak RP-14 films as follows: exposure time 7 days: Kodak Liquid X-ray developer, 5 min.: Kodak Rapid Fixer 5 min.

3. Column Chromatography

4-^{14}C-T and 7α-^{3}H-A tracer for incubation studies were purified on Sephadex LH-20 columns prior to use. An 0.85 x 12 cm column was packed with 1.75 gm LH-20, previously swelled in 20 ml benzene:methanol (17:3). The column was washed with 10 ml benzene:methanol (17:3), followed by 15 ml isooctane:benzene:methanol (18:1:1). 4-^{14}C-T or 7α-^{3}H-A (0.5 - 1.0 μCi) was applied in 0.2 ml eluting solvent to the column and eluted with isooctane:benzene:methanol (18:1:1). Fractions were collected and aliquots (1/100th) were counted in 5 ml toluene containing PPO (5 gm/liter). The fractions corresponding to ^{14}C-T or ^{3}H-A from each separation were combined, dried under nitrogen and used for incubation studies.

4. Gas Chromatography (GC)

A Varian Model 2100 Gas Chromatograph with FID and 4' x 1/8" (I.D.) or 6" x 1/4" (I.D.) glass columns packed with 3% OV-17 on Chromosorb W (HP) 80/100 mesh was used for GC. Conditions for the analysis were: heated injection port, 305°C: FID 305°C: oven 270°C: N_2 carrier gas 40 ml/min. Similarly a 6" x 1/4" (I.D.) column of 1.5% SE-52 on Chromosorb 750, 80/100 mesh was employed: injection port temp. 305°C: FID, 305°C: oven 235°C: N_2 carrier gas 40 ml/min.

For some analyses, retention times were measured relative to testosterone acetate as internal standard. The signal was attenuated at 16×10^{-11} or 16×10^{-10} for all analyses. Sample injection was 0.5 - 1.0 µg in 1 µl methanol. To assure reproducibility of results, columns were periodically conditioned with 3 x 10 µl injections of Silyl-8 and baked at 280°C overnight. The coefficient of variation for retention times on repeated injections was <0.1%.

5. Mass Spectroscopy (MS)

GC-MS was done on a Varian Model 2700 Gas Chromatograph interfaced to a Varian MAT CH7 Mass Spectrometer by a Watson-Biemann separator. Analytical conditions were: column 6' x 1/4" (I.D.) glass 1.5% OV-17 on Chromosorb W (HP) 80/100 mesh; oven 270°C; separator 300°C; ion source 280°C; ionizing voltage 70 eV; emission current 300 µ amp; sample size 1-2 µg. Data were collected and analyzed by an on-line computer. This work was done courtesy of Dr.J.Rosenfeld, Department of Pathology, McMaster University.

Low resolution mass spectra were obtained on a Consolidated Electrodynamics Model 21-110B Mass Spectrometer. The ion source temp. was from 120°C-200°C; ionizing voltage 80 eV. Data were collected graphically and analyzed manually. The assistance of F.Ramalan, Department of Chemistry, McMaster University is gratefully acknowledged.

(c) Derivative Formation

Acetylation was in acetic anhydride:pyridine (1:2) overnight in the dark at room temp., followed by evaporation of solvent under nitrogen with heating. Trimethylsilyl (TMS) derivatives were prepared in N,O-bis(trimethylsily)-trifluoro-acetamide:chloro-trimethylsilane:pyridine (45:5:2) with heating at 80°C for 20 min. Estrogen methyl esters were synthesized by the method of Brown (1955) using dimethyl sulphate and by extractive alkylation (EA) (Daley et al. 1976).

(d) Recrystallization to Constant Specific Activity

Definitive identification and purity testing of microgram quantities of radioactive steroids was achieved by recrystallization to constant specific activity, with carrier following the method of Axelrod et. al. (1965). Unlabelled steroid, previously re-crystallized from methanol and found to be pure as judged by at least two methods of the following: paper chromatography, thin-layer chromatography, gas chromato-graphy or mass spectroscopy, were weighed to the nearest ± 5 µg on a Mettler M-5 microbalance. Sufficient carrier was taken to give approximately 1 mg of carrier for each 2,000 dpm of radioactivity, whenever possible. A minimum of 10 mg carrier was required for each sample and at least 2000 dpm of initial radioactivity was required in a sample

for a minimum of 3 successive recrystallizations to constant specific activity.

Solvent pairs methanol:water, acetone:hexane and acetone:water were used. The sample to be characterized was dissolved in the minimal volume of the first solvent with gentle warming. A few drops of the second solvent were added and the sample was left undisturbed overnight at room temperature or in the cold room to affect crystallization. The mother liquor was separated from the crystals, transferred to a small glass weighing bottle, dried under air or N_2, kept in a dessicator for 1-2 days and weighed. The crystals were allowed to dry in a dessicator for 2-3 days and a portion (1-5 mg) was taken carefully and weighed on the Mettler M-5 balance. A portion of, or in some cases, all of, the mother liquor was also taken and weighed. The weighed crystals and mother liquor were transferred to counting vials and counted for 10 min in 10 ml toluene containing PPO (4 gm/liter) and POPOP (0.1 gm/liter) (toluene/PPO/POPOP).

The remaining crystals were redissolved in solvent pair and recrystallized as above. Mother liquor and crystals were separated and counted. This procedure was repeated 2-3 more times until the specific activity (dpm/mg) in the crystals was constant and to within ± 10%

of the specific activity in the mother liquor. In some cases when the initial radioactivity in the sample was low (<5,000 dpm) identification was achieved when the specific activity of the crystals in the third and fourth recrystallizations did not deviate more than 10% from the average.

(e) Purity of Solvents and Standards

All solvents with the exception of pyridine and diethyl ether were purified by redistillation from glass prior to use. Methanol, benzene and methylene chloride for use in mass spectroscopy were purified in sulphuric acid washed glassware by fractional distillation from glass columns packed with glass O-rings courtesy of Dr.V.Taguchi, Department of Pathology, McMaster University Medical Center.

Purity of all solvents was determined by GC. Solvent (5 ml) was concentrated 100 X and analyzed using a Packard Model 823 GC equipped with FID and a 6' x 1/4" (I.D.) glass column packed with 1.5% OV-17 on Chromosorb W(HP) 80/100 mesh. Conditions for the analysis were as follows: heated injection port, 250°C; FID, 300°C; oven, 270°C; N_2 carrier gas 40 ml/min.

A number of trace impurities were detected in the solvents methylene chloride, diethyl ether, and methanol. These compounds were found by GC and GC-MS to belong to the series of pthalate esters. They were found in unopened stock as well as in redistilled solvents. Curiously, the amounts of these impurities

could be increased 100-200% by allowing the solvent to sit in a bottle left unopened overnight. This may have been the result of contamination via central air-conditioning apparatus as has been suggested by other workers (Canada Center for Inland Waters, unpublished observations). Various washing procedures for the solvents (acid, alkali) were tested but the amounts of contaminating substances were either unchanged or increased. Since the characteristic pthalate ion (m/e 149) is a very stable one, contamination of the GC-MS traces of the estrogens obtained in the benzene:ether (1:1) eluate from extractive alkylation was encountered. However, by using large amounts of estrogen (5 µg) relative to the solvent contaminants, satisfactory GC-MS responses were obtainable.

Purity of all non-radioactive steroids was checked before use by paper and/or thin-layer chromatography. Stock solutions of 50 µg/ml were prepared in methanol and stored at 4°C. The purity and identity of some non-radioactive steroids was also determined by gas chromatography and mass spectroscopy. ^{3}H- and ^{14}C-labelled steroids were stored in benzene:ethanol (9:1) or ethanol at -20°C. Radiochemical purity and identity was checked by paper or thin-layer chromatography before use. If impurities exceeding 3% were detected, the material was purified by paper, thin-layer or

column chromatography prior to use.

(f) Neutral-Phenolic Partitioning

Chemical separation of estrogens from other steroids by phenolic extraction has been widely used in the study of estrogens (McGregor et. al., 1961; Reed et. al., 1972). The toluene-sodium hydroxide partitioning method, modified from Engel et. al. (1950) was used for all incubation studies except the hypothalamus and cortex. Incubation mixtures were extracted with 3 x 10 ml ether. The organic and aqueous phases were separated by centrifugation at 3500 rpm for 5 min. The aqueous layer was discarded or in some experiments an aliquot (2 ml) was counted in 14 ml Triton-X cocktail. The ether was washed with water (10 ml), the aqueous and organic layers separated again by centrifugation and the aqueous discarded. The organic phase was transferred to a 50 ml separatory funnel and dried overnight in the fume hood. Toluene (20 ml) was added and extracted with 1 N NaOH (3 x 10 ml). The NaOH was backwashed with fresh toluene (5 ml) and the toluene was combined and washed with 5 ml water. The aqueous layer was discarded and the toluene was evaporated under a stream of nitrogen and the residue was stored in a desdicator for one to two days before chromatography.

A method for the separation of neutral from phenolic steroids was developed for the purpose of studying the aromatisation of T in the hypothalamus and cortex. Because of the limited conversion, and hence the large amounts of tracer required to

produce detectable amounts of estrogens, a method was needed to separate androgens from estrogens to give the estrogens in a high yield and in a purified form suitable for crystallization. Since crystallization of estrogens as the 3-O-methyl derivatives is easier than crystallization of the parent compound, a method which combined chemical separation of the estrogen from the androgen fraction with subsequent direct methylation of the estrogen fraction to give the methylated derivatives was desirable. A method was developed to satisfy these criteria, using a modification of the "extractive alkylation" (EA) technique (Ervik and Gustavii, 1974). The complete development of the technique is given later in the Experimental section. For the hypothalamus and cortex incubations, the method involved the following: To an ether extract (3 x 10 ml) of the incubation medium and tissue was added toluene (3 ml). The toluene was extracted with 2 x 3 ml. 1 N NaOH on a Burrell mechanical shaker for 10 min. per extraction at a speed setting of 7. The aqueous and organic layers were separated by centrifugation and the NaOH was backwashed with toluene (5 ml). The neutral fraction was not processed any further. The NaOH was used for EA.

To the NaOH was added 100 µl 0.1 M tetrahexylammonium hydroxide (THAH) in methanol and 5 ml 0.5 M methyl iodide in methylene chloride (CH_3I/CH_2Cl_2). The mixture was shaken 10 min, centrifuged, the NaOH discarded and the organic phase containing the estrogen methyl ethers was

transferred to an 18 x 0.4 cm glass column packed with florisil, and previously washed with a minimum of 10 ml methylene chloride. The column was developed with 5 ml methylene chloride. Estrogen methyl ethers were eluted with 10 ml benzene:ether (1:1, v/v) and the eluate was dried under nitrogen and stored at 4°C for chromatography.

EXPERIMENTAL AND RESULTS

(a) Liver Incubations with 7α-^{3}H-Testosterone

Mice were anesthetized with 0.7 ml Avertin (Merck Index), i.p. and decapitated. Livers were rapidly dissected, placed in ice-cold 0.9% saline and freed of adhering tissue. A homogenate of 10-15 mg wet wt. liver per ml was prepared in Medium 199 using a ground glass homogenizer. Aliquots (1 ml) were incubated with 0.4 μC_i 7α-^{3}H-T (25 μC_i/mM) in 25 ml Erlenmeyer flasks in 5 ml Medium 199 containing 0.1 ml propylene glycol for two hours at 37°C in a Dubnoff metabolic shaker (100 oscillations/min) with air as the gas phase. Incubations were terminated by quick freezing and stored at -78°C.

Tissue and medium were extracted with 3 x 10 ml ether, with centrifugation for 5 min at 3500 rpm to separate the phases between extractions. The combined organic extracts were washed with 10 ml water, again with centrifugation at 3500 rpm for 5 min. The aqueous was discarded and the organic was transferred to a 50 ml separatory funnel, and evaporated to dryness under air.

The extract was partitioned between toluene and sodium hydroxide to give neutral and phenolic extracts. A portion of each extract (1/100 and 1/20 respectively) was taken and counted in 10 ml toluene/PPO/POPOP for recovery.

The neutral extracts were transferred in 3 x 10 µl chloroform:methanol (1:1) to 20 x 20 cm sheets of silica gel F-254, 0.25 mm thickness and chromatographed in chloroform: methanol at 4°C. The solvent front was allowed to advance 17 cm from the origin, the sheets were removed from the tank, air dried and rechromatographed in the same direction in benzene:ethyl acetate (13:1 at room temp.) This procedure was found to separate T, A, androstanediol (5α & 5β) (AOH_2), dihydrotestosterone (DHT) (5α & 5β). Unlabelled standards were included in each chromatographic step. The areas corresponding to 5α and 5β AOH_2 were scraped from the thin-layer plate and collected into a Pasteur pipette with a 1-2 cm glass wool plug. The silica gel was eluted with 3 ml methanol and the eluate was collected, dried in 12 x 75 mm disposable glass tubes and transferred to Whatmann #1 papers and chromatographed in Bush B_3. The papers were dried and scanned for radioactivity. The area corresponding to 5α-AOH_2 was cut and eluted with 10 ml methanol. The eluate was collected in a 15 ml glass centrifuge tube dried under air, 2 ml methanol was added, 0.1 ml was taken for counting and the remaining methanol was again removed by drying under air. Carrier 5α-AOH_2 was added to each eluate and recrystallized four times in methanol:water.

The area corresponding to T and DHT was scraped

from the thin-layer plate and eluted with 3 ml methanol as above. The eluate was chromatographed in Bush A and the papers were scanned for radioactivity. Zones of radioactivity were cut and eluted with 10 ml methanol as above. T and 5α-DHT were crystallized from methanol: water.

The area corresponding to A was scraped from the thin-layer plate, eluted with 3 ml methanol as above and chromatographed in Bush A. Papers were scanned for radioactivity and the area corresponding to A was cut and eluted in 10 ml methanol, dried under air, an aliquot (1/20) counted. Final identification of A was achieved by recrystallization from acetone:hexane.

No internal standards were included for correction of losses in the experimental procedures used. To determine the efficiency of extraction of various steroids from liver homogenates, liver homogenates from BALB/c ♂ mice were prepared as described above. Homogenates (1 ml) were mixed with 5 ml Medium 199 and 0.1 ml propylene glycol containing tracer amounts of (1) 7α-^{3}H-T, (2) 4-^{14}C-A, (3) 4-^{14}C-5α-DHT or (4) 4-^{14}C-5α-AOH_2. Four of each were done. The mixture was extracted with 3 x 10 ml ether as above and the ether extracts were dried in scintillation vials, dried under nitrogen and counted in 10 ml toluene/PPO/POPOP.

To determine if significant amounts of radioactivity remained in the aqueous phase after ether extraction, 8 aliquots

of liver homogenates from BALB/c mice were incubated with 0.4 μC_i 7α-^{3}H-T as above for 2 hr. The tissue and medium were extracted again as above and the remaining aqueous phase was decanted from each incubation, an aliquot was taken (2 ml) and counted in 14 ml Triton-X cocktail. The percentage of radioactivity added as ^{3}H-T remaining in the aqueous phase after extraction was calculated.

The rate of disappearance of T and the appearance of A was determined in one experiment in which liver homogenates (1 ml) in duplicates from BALB/c mice were incubated as described above for varying periods of time, up to 2 hr. T and A were determined in each incubation as described above.

A total of 14 liver incubations of normal (BALB/c) and Tfm mice (o^+ and o^{hv}) were analyzed. 11-14 mg wet wt liver tissue was used for each incubation. There were no significant differences between the amounts of tissue used per incubation for each of the three genotypes used. Following ether extraction and toluene sodium-hydroxide partitioning, the major portion (ave. 73%) of the added radioactivity was found in the neutral fraction. There were no significant differences between the radioactivity in the neutral fractions in the three groups (Table 8). A small (<1%) amount of radioactivity was recovered from the phenolic fractions of each incubation. Significant differences were found between

the percentage of radioactivity in the phenolic fractions when comparing BALB/c with Tfm (o^+) ($p < 0.025$) and BALB/c with Tfm (o^{hv}) ($p < 0.001$). There were no differences between Tfm (o^+) and Tfm (o^{hv}).

Small amounts of radioactivity corresponding to 5α-AOH_2 were recovered from the incubations of BALB/co but not Tfm (o^+) or Tfm (o^{hv}) (Fig.3). 5^{α}-androstanediol was identified in incubations of BALB/c liver after recrystallization with carrier (Table 5). The percentage conversion was low in each incubation ($< 0.5\%$). The areas corresponding to T was chromatographed in Bush A and three areas of radioactivity corresponding to polar compounds at the origin, a second area corresponding to DHT and a third area corresponding to T were found in incubations of normal and Tfm livers as shown in the paper chromatography scans in Fig. 4. The polar compound was not studied any further. The area corresponding to DHT (10-20% of the added radioactivity) was eluted from each incubation and crystallized with carrier. After 2 or 3 recrystallizations, no radioactivity remained in the crystals indicating that no DHT was formed. The identitity of this region was not established. The area corresponding to T was recrystallized to constant specific activity (Table 6). Rechromatography of the region corresponding to A gave only one compound after paper scanning (Fig.5). This material was eluted and recrystallized with carrier to constant specific

activity (Table 7).

The percentage recovered as T and A from each incubation was determined from the radioactivity crystallized to constant specific activity with carrier, calculated as the average of the n-1 and n recrystallizations for each identification, expressed as a percentage. This percentage was multiplied by a factor to correct for small differences in the amount of tissue used for each incubation. The final results are expressed as a percentage per 10 mg tissue per 2 hours as shown in Table 8. The conversion of T to A was similar in BALB/c and Tfm (o^+) and approximately twice that of the conversion of T to A in the Tfm (o^{hv}) incubations. Expressed as a ratio (A/T), the results show differences in the metabolism of T in all three genotypes studied, with the formation of products other than A being most extensive in the Tfm (o^+) incubations.

The recoveries of tracer amounts of 7α-^{3}H-T, 7α-^{3}H-A, 4-^{14}C-DHT and 4-^{14}C-AOH$_2$ in ether extracts were 90.7 ± 0.8% (S.E.), 93.4 ± 0.9%, 97.1 ± 0.8% and 96.4 ± 1.1% respectively. Thus there were no differences in the initial extraction which could have accounted for differences in T and A recovered from each incubation. A small amount (1.3% ± 0.09, n = 8) of the radioactivity remained in the aqueous phase of 2 hour incubations of BALB/c liver, after ether extraction, indicating that in the system used, steroid

FIGURE 3: Paper chromatography of 5α-androstanediol from liver incubations with 7α-^{3}H-testosterone

Solvent system Bush B_3

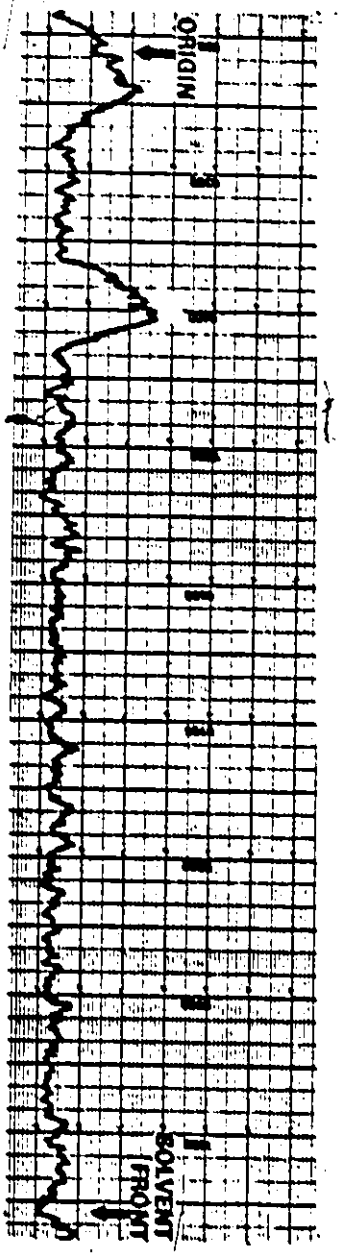


FIGURE 3

FIGURE 4: Paper chromatography of testosterone from liver incubations with 7α-^{3}H-testosterone

Solvent system Bush A

(a) BALB/c

(b) Tfm (o^{+})

(c) Tfm (o^{hv})

I origin

II unidentified

III testosterone

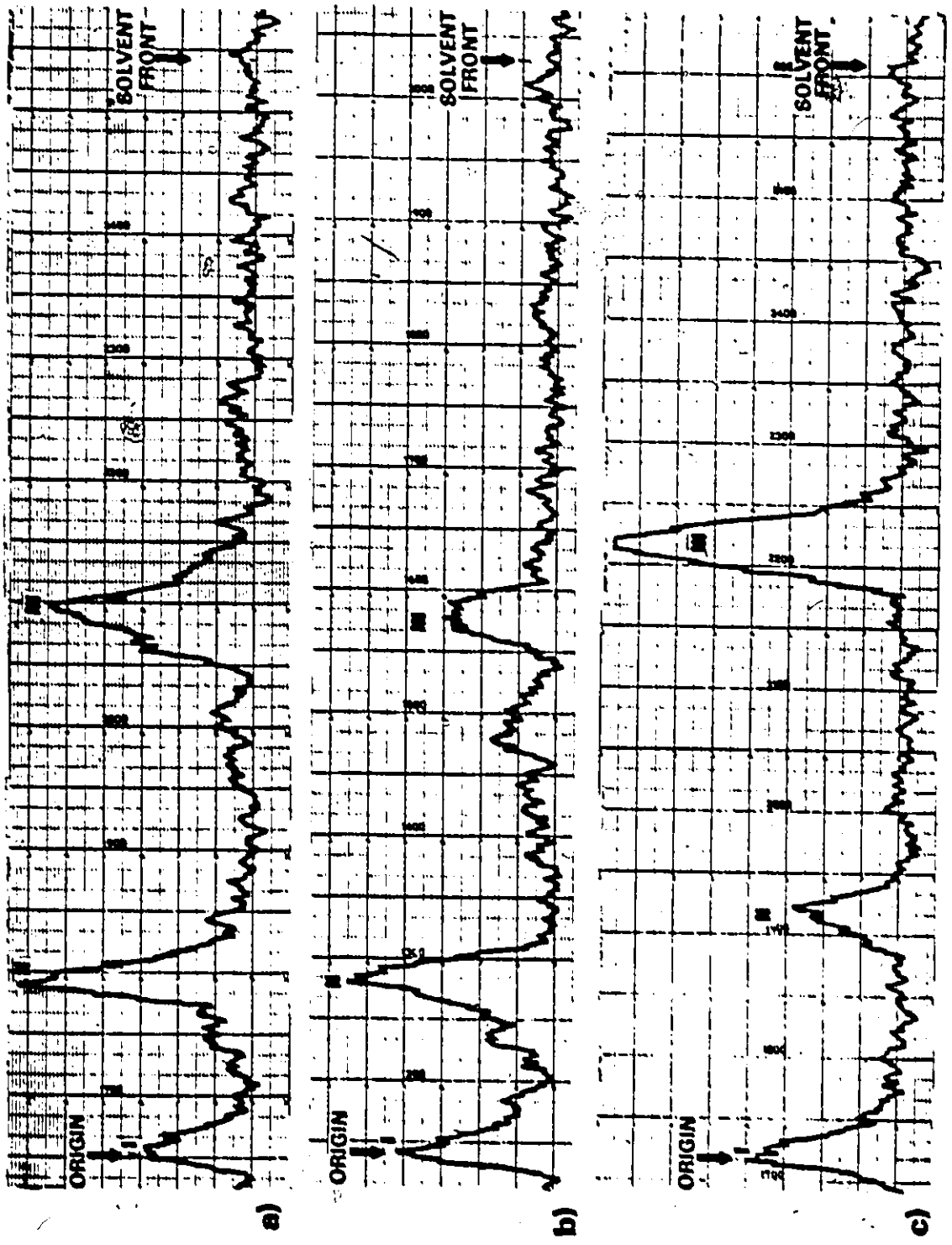

ORIGIN
SOLVENT FRONT
a)
ORIGIN
SOLVENT FRONT
b)
ORIGIN
SOLVENT FRONT
c)

FIGURE 4

FIGURE 5: Paper chromatography of androstenedione from liver incubations with 7α-^{3}H-testosterone

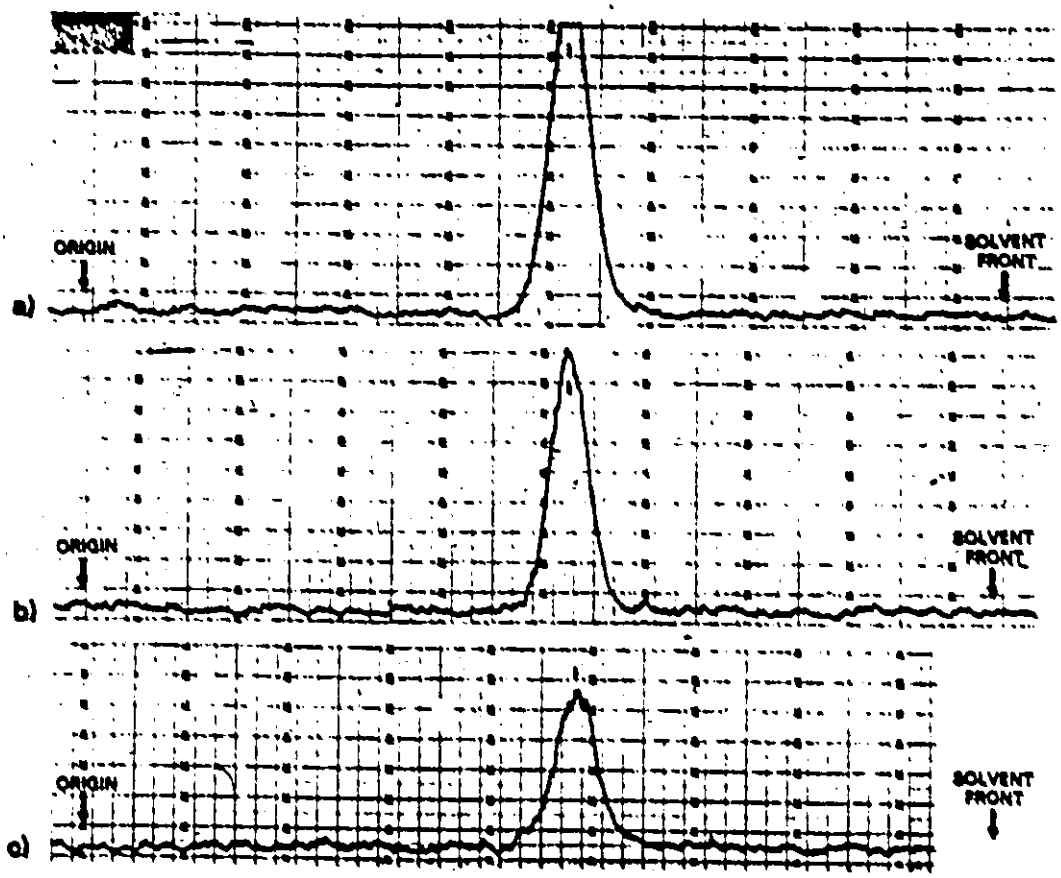
Solvent system Bush A

(a) BALB/c ♂

(b) Tfm (o^{+}) ♂

(c) Tfm (o^{hv}) ♂

I androstenedione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ORIGIN
SOLVENT FRONT
c)

FIGURE 5

FIGURE 6: Change in ^{3}H-androstenedione/^{3}H-testosteone ratio with time in liver incubations with 7α-^{3}H-testosterone

Conditions as described in text.
BALB/c ♂

Each point is the mean of duplicate determinations.

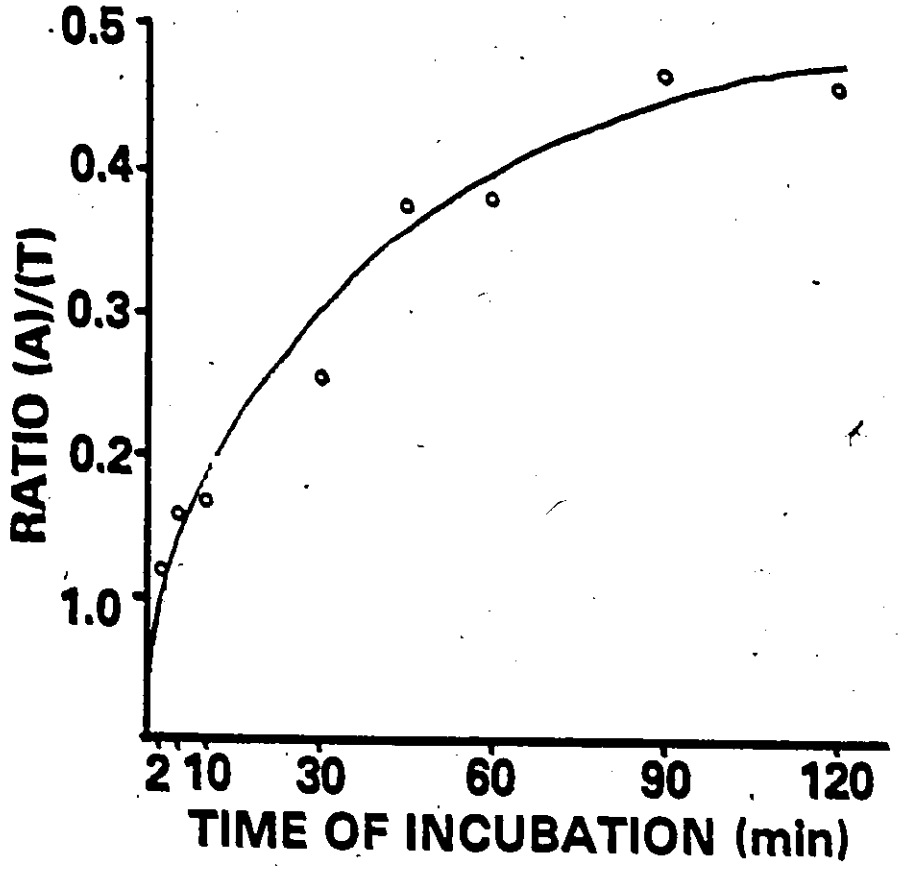


FIGURE 6

Table 5. Recrystallization of ^{3}H-5α-androstenediol from liver incubations with 7α-^{3}H-testosterone.

genotype	mg carrier	specific activities (cpm/mg)+ n-3	n-2	n-1	n
BALB/c	7.52	227/3292	206/2021	122/597	114/236
	8.82	219/1130	246/931	109/301	121/221
	9.03	219/2269	210/-	109/452	112/218
Tfm (o^{+})	23.45	13/460	*/134	8/*	*/*.
	25.88	63/30	59/11	54/*	-/*
Tfm (o^{hy})	24.73	65/457	*/190	*/50	*/*
	18.40	30/124	15/138	10/54	*/20

\+ n denotes number of successive recrystallizations (4), upper figure denotes crystals, lower figure denotes mother liquor.
* no radioactivity above background
\- sample lost

Table 6. Recrystallization of ^{3}H-testosterone from liver incubations with 7α-^{3}H-testosterone

genotype	mg carrier	specific activities (cpm/mg)+ n-3	n-2	n-1	n
BALB/c ♂	42.48	828/1959	811/889	815/845	814/820
	45.35	754/1282	785/964	732/760	751/750
	46.29	991/1623	954/1039	970/1026	960/981
Tfm (o^{+}) ♂	37.71	120/197	122/157	119/136	120/126
	22.80	175/205	111/135	103/104	105/102
Tfm (o^{hv}) ♂	46.50	228/643	226/600	215/350	220/242
	45.86	314/410	300/385	275/290	284/271

Table 7. Recrystallization of ^{3}H-androstenedione from liver incubations with 7α-^{3}H-testosterone

genotype	mg carrier	specific activities (cpm/mg)+ n-3	n-2	n-1	n
BALB/c ♂	50.22	3953/5277	4113/4082	3996/3981	4020/4016
	35.18	3842/5772	3791/4135	3839/3858	3840/3800
Tfm (o^{+}) ♂	51.89	670/955	667/715	679/681	634/659
	53.03	342/598	324/293	297/301	307/290
Tfm (o^{hv}) ♂	41.63	607/789	324/510	291/317	320/352
	50.51	386/821	380/454	372/411	357/390

+ n denotes number of successive recrystallizations (4-6) upper figure denotes crystals, lower figure denotes mother liquor.

Table 8. 7α-^{3}H-Testosterone metabolism in liver homogenates.

genotype	n	mg tissue	% recovered neutral	% recovered phenolic	% recovery of (T)*	% conversion to (A)*	A/T
BALB/c ♂	6	13.2 ± 0.4	70.9 ± 2.4	0.4 ± 0.3	11.4 ± 0.8	50.5 ± 2.5	4.5
Tfm^{+}(o^{+})+/Y♂	4	12.8 ± 0.7	71.9 ± 1.1	1.4 ± 0.1	6.1 ± 0.5	47.5 ± 1.0	7.8
Tfm^{+}(o^{hv})Blo/Y♀	4	13.5 ± 0.3	77.1 ± 2.9	1.1 ± 0.2	18.5 ± 1.0	25.7 ± 1.0	1.4

* results are expressed as percent per 10 mg tissue per 2 hr (mean ± S.E.M.). All differences are significant (p 0.001) except for A in BALB/c and Tfm (o^{+}) $p < 0.005$ (Student's "t" test)
+ means ± S.E.M.
n = number of incubations

conjugation was minimal.

The formation of A from T as a function of time in the series of liver incubations described above is shown in Fig. 6. The results are expressed as the ratio of A/T. The A/T begins to plateau at approximately 90 min. and levels off between 90 min. and 120 min. to reach a value similar to the result obtained from the 6 BALB/c incubations for two hours.

After phenolic partitioning and chromatography of the phenolic extracts in Tol/PE, no radioactivity above background was detected on scanning of the paper chromatographs. Areas corresponding to E_1 and E_2 standards were cut, eluted with 10 ml methanol and counted in 10 ml toluene/PPO/POPOP. After correction for background, small amounts of radioactivity (260-440 dpm) were found in the region corresponding to E_1 and smaller amounts (50-100 dpm) of radioactivity were found associated with E_2. These very small amounts of radioactivity could not be identified by recrystallization.

(b) Testes Incubations with 7α-^{3}H-Testosterone and 4-^{14}C-Progesterone

Testes were decapsulated and the tunica albuginea discarded. The remaining tissue was minced finely with small scissors, blotted with tissue paper and weighed. Tissue minces (10-28 mg wet wt.) were incubated in 5 ml Medium 199

with 0.2 μC_i 4-^{14}C-P (52.8 mC_i/mM) and 0.5 μC_i 7α-H-T (25 C_i/mM) dissolved in 0.1 ml propylene glycol for 3 hours in a Dubnoff metabolic shaker at 100 oscillations/min at 37°C with air as the gas phase. Incubations were terminated by quick freezing and stored at -78°C or extracted immediately. Tissue and medium were extracted with 3 x 10 ml ether with centrifugation at 3500 rpm for 5 min between extractions to separate aqueous from organic phases. The organic extract was dried under nitrogen, an aliquot (1/100) counted and the extract was partitioned between toluene and sodium hydroxide to give neutral and phenolic fractions. Aliquots of the neutral (1/100) and phenolic (1/20) were counted in 10 ml toluene/PPO/POPOP. The remaining neutral extract was evaporated to dryness and transferred in 3 x 10 µl of chloroform:methanol (1:1) to Whatman #1 chromatography paper and developed in Bush A. After radioactive chromatogram scanning, all areas of radioactivity were cut and eluted with 10 ml methanol. Radioactivity corresponding chromatographically to: (1) T, 17α-hydroxyprogesterone, (17α-OHP), 5α-AOH_2, 5β-AOH_2, (2) A, DHT and androsterone, (3) P, were rechromatographed in the chromatographic systems (1) TLC-C, (2) TLC-B, (3) Bush B_3 respectively. TLC-C separated (1) into two zones corresponding to 17α-OHP and T and 5α and 5β-AOH_2. Aliquots (1/20) of all radioactive zones were counted. The chromatographically pure zones corresponding to T,

5α-DHT, A and P were then recrystallized to constant specific activity.

The phenolic fractions were treated as follows. After counting an aliquot from each, the remainder was taken to dryness under nitrogen and then transferred in 3 x 10 µl chloroform:methanol (1:1) to Whatman #1 papers and run in Tol/PE. All strips were scanned for radioactivity and areas corresponding to E_1 and 17β-E_2 were cut, eluted and counted in 10 ml toluene/PPO/POPOP.

A total of ten testes incubations with ^3H-T and ^{14}C-P were performed. After extraction and neutral phenolic partitioning there were no significant differences in the percentage recovery of ^3H or ^{14}C radioactivity in either the neutral or phenolic fractions. Radiochromatogram scans of the neutral extracts in Bush A are shown in Fig. 7. After further chromatography as described above, major zones of radioactivity corresponding to T, A and P were crystallized to constant specific activity with the appropriate carrier (Table 10). From the average of the specific activity in the final two crystallizations, the percentages recovered were expressed as radioactivity crystallized with carrier, divided by the total radioactivity recovered from the final chromatography steps. Corrections were made for the small variations in tissue weights used in different incubations

by dividing the percentage of each compound by the number of mg tissue incubated and multiplying by ten to give the percentage recovered per ten mg of tissue per three hr.

The results are shown in Table 11. While there were no differences in the percentages of T conversion to A or the percentage recovered as unchanged substrate there were differences in the pattern of P metabolism. A formed from P accumulated to a greater extent in incubations of Tfm (o^+) testes than in normal BALB/c testes. Similarly, there was a greater amount of A found in the Tfm (o^{hv}) incubations than in BALB/c. However less A was formed from P in Tfm (o^{hv}) than in Tfm (o^+) incubations.

The recovery of radioactivity in the phenolic fractions as a percentage of the total radioactivity added was low (1.5%). However, there were significant differences between the recovery in Tfm (o^+) and Tfm (o^{hv}) incubations, compared to the BALB/c incubations. Paper chromatograph scans of the Tfm (o^+) and Tfm (o^{hv}) incubations showed a large peak (approx. 10,000 dpm ^3H and 10,000 dpm ^{14}C) of radioactivity with R_f 0.9 (Fig.8). Chromatographically, this material did not correspond to E_2 or E_3. The R_f was similar to that of E_1, but after crystallization of E_1 in methanol: water, all the radioactivity went into the mother liquor. This material was not found in incubations of BALB/c testes or control

FIGURE 7: Paper chromatography of neutral steroids extract from testes incubations with 7α-^{3}H-testosterone and 4-^{14}C-progesterone

Solvent system Bush A

(a) BALB/c ♂

(b) Tfm (o^{+}) ♂

(c) Tfm (o^{hv}) ♂

I origin

II T, 17OHD, 5αAOH$_2$

III unidentified

IV A

V P

VI unidentified

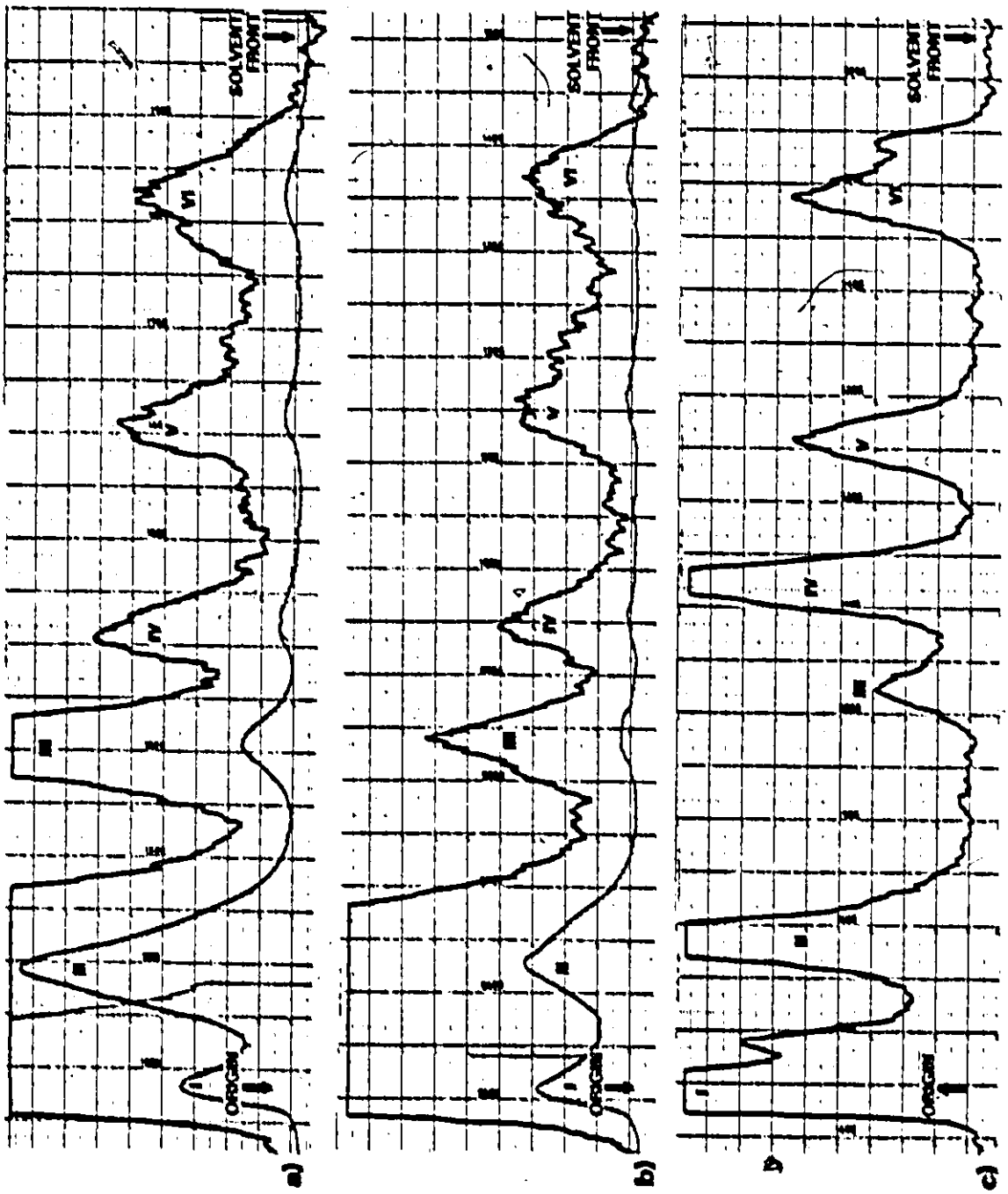


FIGURE 7

FIGURE 8: Paper chromatography of phenolic steroids extract from testes incubations with 7α-^{3}H-testosterone and 4-^{14}C-progesterone

Solvent system Tol/PE

(a) Tfm (o^{+}) ♂

(b) Tfm (o^{hv}) ♂

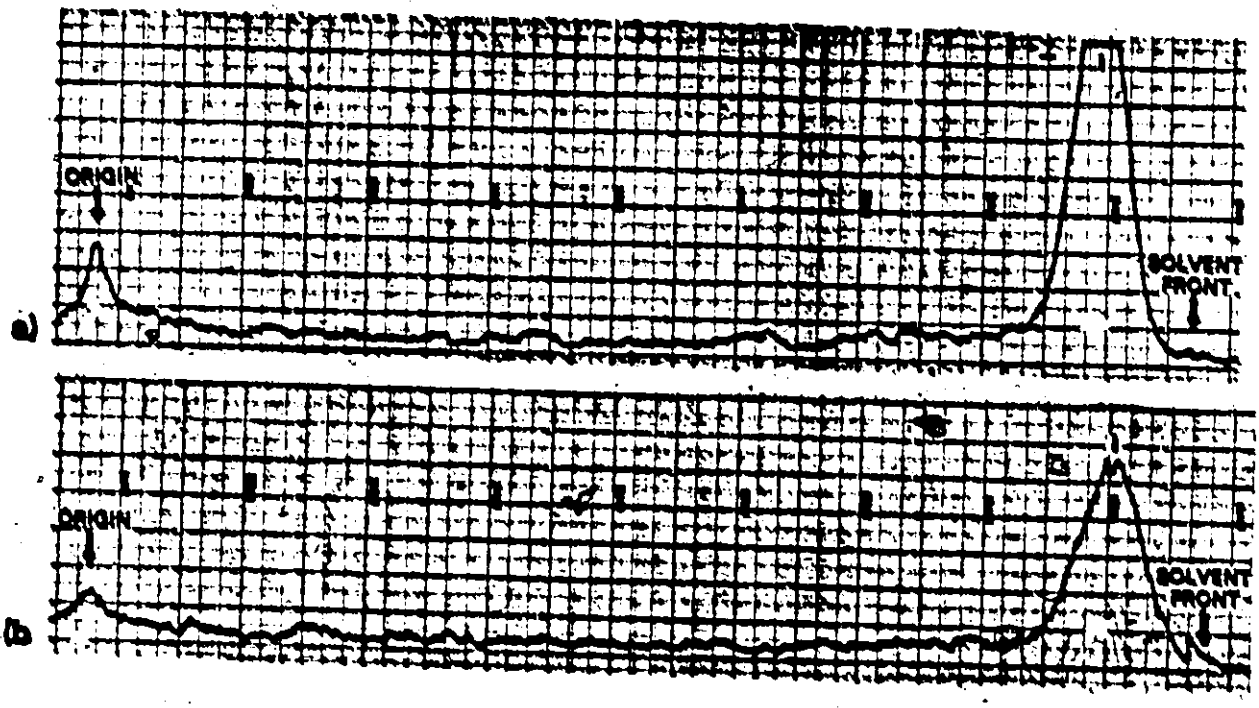


FIGURE 8

Table 9. Recovery of ^{3}H- and ^{14}C- in testes incubations with 7α-^{3}H-testosterone and ^{14}C-progesterone

genotype	n	mg tissue	neutral		phenolic	
			^{3}H	^{14}C	^{3}H	^{14}C
BALB/c ♂	6	19.2 ± 2.2	76.6 ± 1.1	72.1 ± 1.5	1.4 ± 0.1	0.5 ± 0.1
Tfm (o^{+}) ♂	4	13.0 ± 2.3	80.5 ± 0.9	74.9 ± 0.4	1.9 ± 0.3	0.2 ±
Tfm (o^{hw}) ♂	4	23.6 ± 1.9	74.4 ± 0.8	70.4 ± 0.5	1.7 ± 0.3	0.4 ± 0.4

means ± S.E.M.
differences between genotypes not significant

Table 10. Recrystallization of ^{3}H- and ^{14}C-testosterone from testes incubations with 7 -^{3}H-testosterone and 4-^{14}C-progesterone

genotype	mg carrier	specific activity (cpm/mg)+ ^{3}H n - 3	n - 2	n - 1	n	^{14}C n - 3	n - 2	n - 1	n
BALB/c ♂	42.25	1949/4150	1780/3020	1695/1980	1700/1710	1256/2571	880/1000	732/887	741/745
	38.90	1740/2551	1656/2301	1650/1931	1671/1850	998/1301	621/700	550/640	568/581
	42.48	1347/2525	1305/1961	1300/1351	1304/1321	932/1334	799/1037	802/745	795/787
	43.83	1009/2003	947/983	823/851	850/860	574/1259	526/721	544/581	535/582
Tfm (o^{+}) ♂	32.05	6300/9421	6287/7200	6055/6200	6045/6205	95/221	92/110	80/90	76/82
	28.57	3521/4457	3500/3992	2308/2400	2200/2421	94/112	82/102	70/84	70/76
Tfm (o^{hv}) ♂	37.98	3900/6245	3010/5030	2895/3100	2900/2995	303/473	275/386	195/225	204/211
	30.39	4235/5287	3998/4980	3846/4120	3890/4100	399/621	302/500	279/349	290/300

+ n denotes number of successive crystallizations (4) upper figure denotes crystals, lower figure denotes mother liquor.

Table 11. 7α-^{3}H-Testosterone and 4-^{14}C-progesterone metabolism in testes incubations

genotype	n	mg tissue	% recovery of (T) ^{3}H	% recovery of (T) ^{14}C	% recovery of (A) ^{3}H	% recovery of (A) ^{14}C	% recovery of (P) ^{14}C	(P)/(T)	P/A
BALB/c ♂	6	19.2 ± 2.2	25.4 ± 2.8	11.0 ± 1.3	1.5 ± 0.8	0.7 ± 0.1	23.3 ± 2.8	2.1 ± 0.6	33.3 ± 3.1
Tfm+(o^{+})+/Y ♂	4	13.0 ± 2.3	26.2 ± 1.9	0.8 ± 0.2	0.8 ± 0.1	7.6 ± 0.8	34.3 ± 2.3	42.9 ± 3.9	4.5 ± 0.5
Tfm+(o^{hx}) Hlo/Y ♂	4	23.6 ± 1.9	19.3 ± 2.3	2.2 ± 0.8	0.8 ± 0.2	1.3 ± 0.2	13.3 ± 1.6	6.0 ± 0.8	10.2 ± 1.0

means ± S.E.M.
results are expressed as percent per 10 mg tissue per 3 hr.
n = number of incubations

incubations without tissue.

(c) Testes Incubations with ^{14}C-Progesterone

Animals were anesthetized with 0.7 ml Avertin i.p. and killed by cervical dislocation. Testes were rapidly dissected and minced finely with scissors. Tissue minces (28-46 mg wet wt.) were incubated with 0.2 μC_i 4-^{14}C-P (52.8 mC_i/mM) in 25 ml Erlenmeyer flasks containing 5 ml Medium 199 with 0.1 ml proplene glycol for 3 hr at 37°C in a shaking water bath at 100 oscillations/min with air as the gas phase. Incubations were terminated by quick freezing and stored at -78°C until extraction. Tissue and medium were extracted with 2x10 ml ether followed with 2x10 ml ethyl acetate with centrifugation at 3500 rpm for 5 min between extractions to separate aqueous from organic. The combined organic extracts were evaporated to dryness and an aliquot (1/100) was taken for counting in 10 ml toluene/PPO/POPOP. The extract was transferred to a 20 x 20 cm silica gel F-254 plate, previously washed in TLC-A and chromatographed in TLC-B at 4°C, dried and rechromatographed in TLC-D in the same direction at room temp. Plates were then autoradiographed for 7 days. Areas corresponding to 5α-AOH_2, 5α-DHT, A, P and two unidentified zones were scraped from the thin-layer plates and eluted with 3 ml methanol from Pasteur pipettes. The area corresponding chromatographically to T and 17α-OHP was eluted and rechromatographed in TLC-C, previously found

to separate T from 17α-OHP. Radioactivity corresponding chromatographically to 5α-AOH_2, 5α-DHT, T, A and P was crystallised to constant specific activity with carrier as before. From the average of the specific activity of the last two recrystallisations, the percentage conversion was determined by dividing the radioactivity in the crystals by the total radioactivity recovered from all areas recovered from the thin-layer plate scrapings, including radioactivity at the origin. To correct for differences in percentage conversion due to variation in the amount of tissue used in each incubation, the conversions were divided by the mg tissue used in each incubation and multiplied by ten. Results are expressed as percentage conversion/10 mg tissue/3 hr.

After the first chromatographic step, 58-70% of the radioactivity added as ^{14}C-P was recovered from elution of all areas of radioactivity shown in Fig.9. The area corresponding to the origin was eluted and counted. Eight major areas of radioactivity corresponding T, A, 5α-DHT, P and three areas of unidentified radioactivity were present in autoradiographs of BALB/cd♂, Tfm + (c^{hv}) Rlo/+++, sxr ♂ and+Ta ++/+++, sxr ♂ incubations. The region closest to the origin was shown to contain small amounts of 5α-AOH_2 by recrystallisation. Similarly, T, A, 5 -DHT and P were identified in all incubations by recrystallisation.

FIGURE 9: Thin-layer chromatography of organic extract from testes incubations with 4-^{14}C-progesterone

Solvent system 1. chloroform:methanol 98:2 (4°C)

2. benzene:ethyl acetate 13:1 (22°C)

(a) Tfm+(o^{hv}) Blo/+++, sxr/+ ♂

(b) +Ta++/+++, sxr/+ ♂

(c) BALB/c ♂

i origin

ii 5α,5β-AOH_2

iii T

iv A

v DHT

vi P

vii) viii) unidentified metabolites

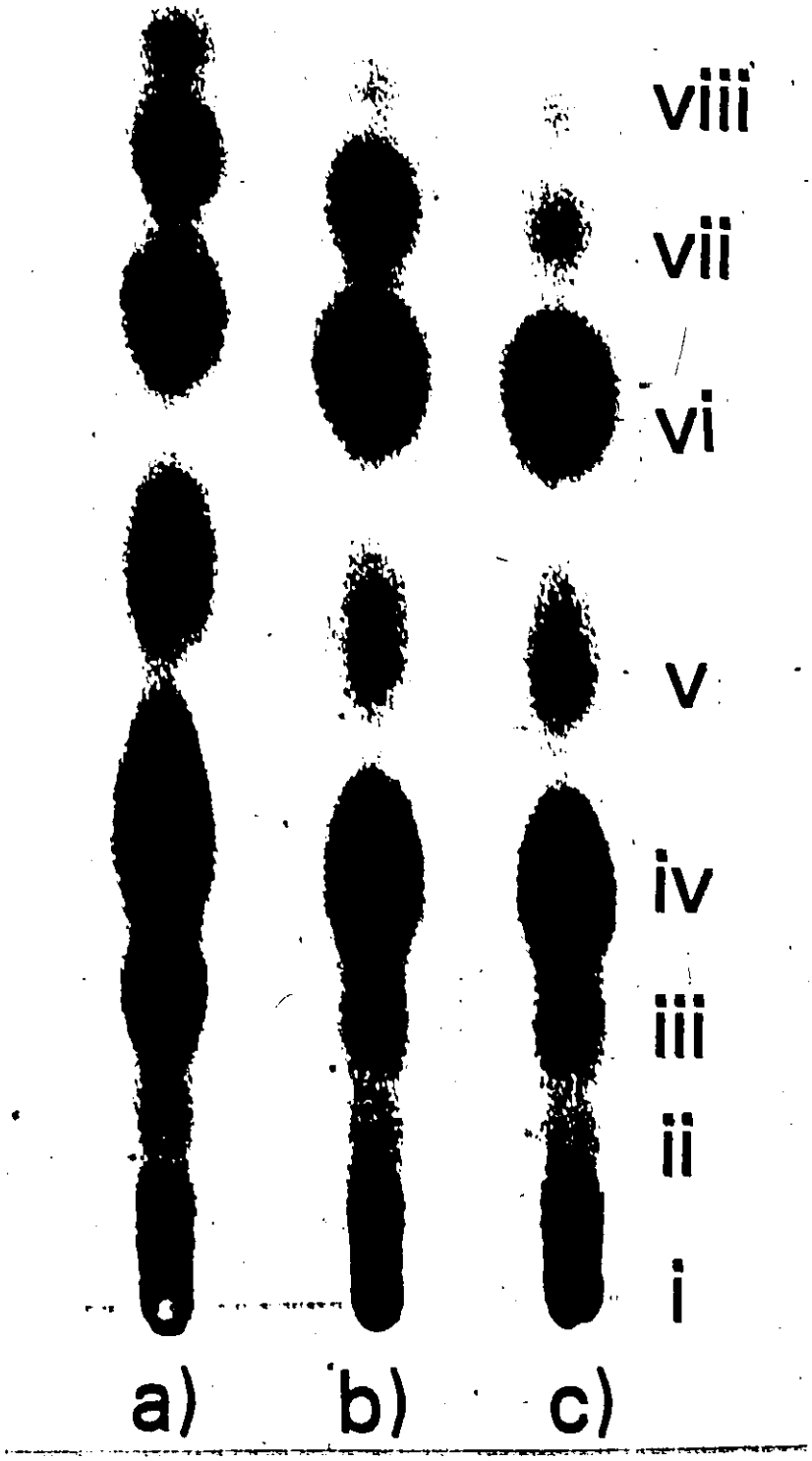


FIGURE 9

Table 12. 4-^{14}C-Progesterone metabolism in testes incubations.

genotype	n	mg tissue	% recovery as P	% conversion to T	A	DHT	AOH_2
BALB/c ♂	4	34.3 ± 2.6	8.6 ± 1.0	4.8 ± 0.6	1.3 ± .1	2.8 ± .1	0.08
+Ta++/+++, sxr ♂	4	31.6 ± 1.9	12.5 ± 1.6	2.8 ± 0.3	3.2 ± .3	1.5 ± .2	0.08
Tfm+(o^{hw})Blo/ +++,sxr ♂	4	37.7 ± 2.7	10.4 ± 1.1	1.9 ± 0.2	6.5 ± .4	1.1 ± .1	0.09

means ± S.E.M.
results are expressed as percent per 10 mg tissue per 3 hr.
n = number of incubations

The formation of 5α-AOH_2 was similar in all incubations and the yield of T and 5α-DHT was similar in all three types of incubations (Table 12). There was more (5 x) A in incubations of Tfm + (o^{hv}) Rlo/+++, sxr ♂ testes than in the BALB/c♂ testes and more (2.5 x) in the incubations of + Ta ++/+++, sxr, ♂ than in the BALB/c♂. The yield of unchanged P substrate was similar in all incubations.

(d) Testes Incubations with 7α-^{3}H-Androstenedione

Four incubations with 7α-^{3}H-A were performed. One mouse of genotype Tfm (o^+) (53 days old) and 3 mice of genotypes Tfm (o^{hv}) (167 days) were used. Mice were anesthetized with 0.7 ml Avertin and decapitated. The testes were rapidly dissected out, decapsulated and minced in ice-cold 0.9% saline. Tissue minces were weighed and incubated with 1 μCi, 7α-^{3}H-A in 5 ml Medium 199 containing 0.1 ml propylene glycol in 25 ml Erlenmeyer flasks in a shaking water bath at 100 oscillations/min at 37°C for 3 hours with air as the gas phase. Incubations were terminated by quick freezing and stored at -78°C. Tissue and medium were extracted with 3 x 10 ml ether, and the ether extract partitioned between toluene and sodium hydroxide in the regular manner. The neutral extracts were chromatographed in Bush B_3 and scanned for radioactivity. The major peak of radioactivity in the Tfm (o^+) incubation corresponded chromatographically to A and was eluted and crystallized with carrier to constant specific activity.

Similarly, radioactivity corresponding chromatographically to T was identified and quantitated in all incubations by recrystallisation. The material at the origin, corresponding to unknown polar material, and a small amount of radioactivity at the solvent front was eluted and counted. From the total radioactivity recovered from the chromatographs, T and A crystallised with carrier were expressed as percentages per 10 mg tissue per 3 hour incubation as before.

In incubations of Tfm (o^{hv}) testes, four peaks of radioactivity were seen in addition to the major one corresponding to A (Fig. 10). A and T were eluted and identified by recrystallisation. The other peaks of radioactivity (R_f 0.09, 0.24, 0.55, 0.81) were eluted and counted. As described above, T and A crystallised with carrier were expressed as percentages per 10 mg tissue per 3 hours.

The results are shown in Table 13. There was no difference between the amounts of T formed in the Tfm (o^{+}) and Tfm (o^{hv}) incubations. In the three Tfm (o^{hv}) incubations, four products in addition to T were found which were not found in the Tfm (o^{+}) incubation. The phenolic extract from each incubation (6-10% of added radioactivity) was chromatographed on Whatman #1 paper in Tol/PE. Radioactivity (R_f 0.16-0.18) was detected in scans of both Tfm (o^{+}) and Tfm (o^{hv}) incubations (Fig. 11). This material (9,000-10,000 cpm) did not crystallise with Ey and further characterisation was not attempted.

FIGURE 10: Paper chromatography of neutral steroids extract from testes incubations with 7α-^{3}H-androstenedione

Solvent system Bush B_3

(a) Tfm (o^{+}) ♀♂

(b) Tfm (o^{hv}) ♀♂

(a) I origin
II T
III A

(b) I origin
II unidentified
III unidentified
IV T
V unidentified
VI A
VII unidentified

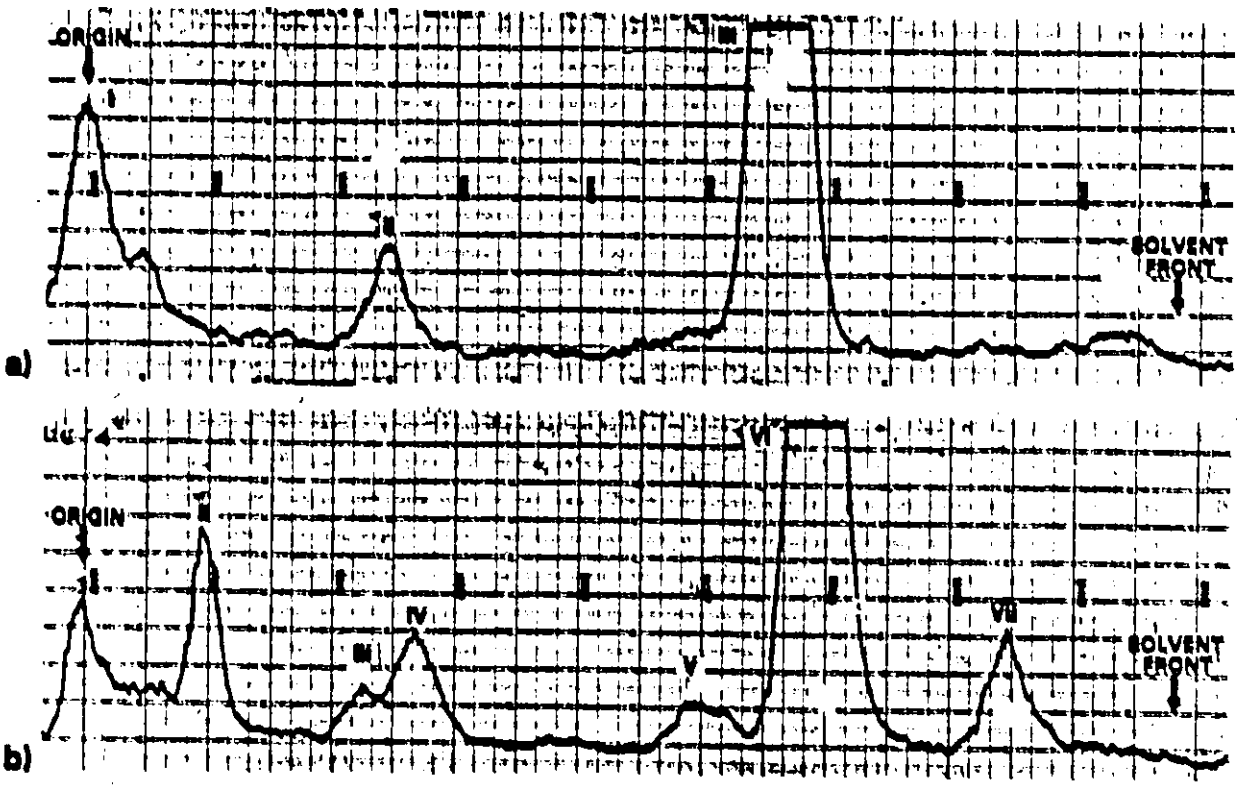


FIGURE 10

Table 13. 7α-^{3}H-Androstenedione metabolism in testes incubations.

genotype	n	% recovery as A	% conversion to T
Tfm(o^{+})♂	1	82.5	6.3
Tfm(o^{hy})♂	3	51.7	5.8

results are expressed as percent per 10 mg tissue per 3 hr.
n = number of incubations

FIGURE 11: Paper chromatography of phenolic steroids extract from testes incubations with 7α-^{3}H-androstenedione

Solvent system Tol/PE

Tfm (o^{+}) ♂

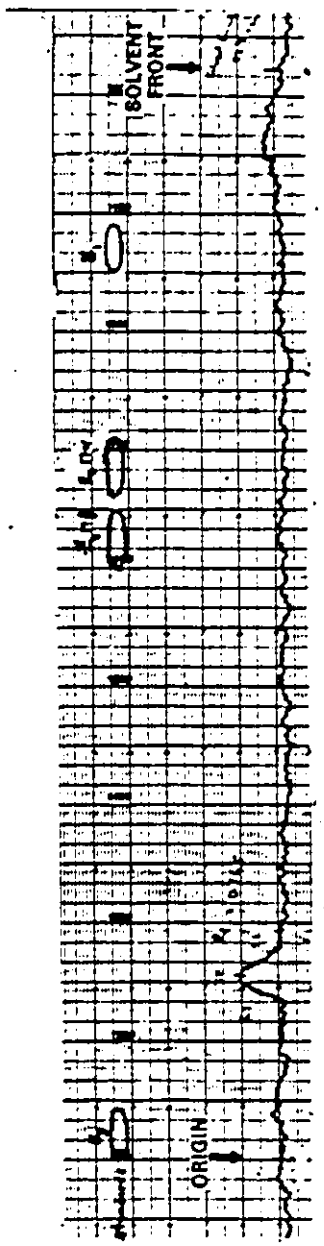


FIGURE 11

(e) Extractive Alkylation

The aromatization of androgens in central nervous tissue has been studied by Naftolin et. al. (1975). Their method involves the incubation of relatively large amounts of tissue (>500 mg) with radioactive androgen and identification and quantitation of the estrogen formed by neutral-phenolic partitioning, chromatography, derivative formation and recrystallization. Because of the large number of steps involved in this procedure, the overall recovery is low (Naftolin, 1975). To determine aromatization in the small amounts of tissue available for the present study (<100 mg), greater overall recovery was necessary to detect estrogen formation within the limits of detection of the experiment. To solve this problem, a rapid and quantitative method for the separation of estrogens from androgens to give the estrogens directly as the methylated derivatives was developed, based on toluene-sodium hydroxide partitioning and direct derivatization of the alkaline extract with CH_3I and THAH in CH_2Cl_2 as follows.

To 0.05-1.0 mg of estrone (E_1), estradiol-17β (E_2-17β), estradiol-17α (E_2-17α), or estriol (E_3) was added 6 ml 1N NaOH. For recovery studies, 1.8×10^{-3} μC_i each of ^{3}H-E_1, ^{3}H-E_2, or ^{3}H-E_3 was added . 1 M THAH (50 µl) and 0.5 M CH_3I/CH_2Cl_2 (5 ml) and the mixture was shaken for 10 min.

The organic and aqueous phases were separated by centrifugation and the organic phase was dried under nitrogen and used for GC. GC showed that EA of E_1, E_2-17β or E_2-17α gave only single peaks with retention times corresponding to authentic estrone-3-methyl ether (E_1OMe), estradiol-17β-3-methyl ether (E_2OMe-17β), or estradiol-17α 3-methyl ether (E_2OMe-17α) (Table 14, Fig.12). Further analysis by TLC and PC confirmed that EA of E_1 and E_2 gave the corresponding 3-methyl ethers (Table 15).

Florisil column chromatography removed the tetrahexylammonium iodide, a yellow solid which was found to interfere with mass spectroscopy and quantitative transfer for paper or thin-layer chromatography. The methylene chloride extract from EA (5 ml) was transferred to florisil columns and eluted with 10 ml benzene:ether (1:1). Further elution with ether (10 ml) and ethyl acetate (10 ml) did not remove any further material from the columns.

Fraction collection (20 drops/fraction) of the benzene: ether eluate showed that E_1OMe and E_2OMe eluted as a single peak. Separation of E_1OMe from E_2OMe on florisil was further attempted by successive elution of the organic extract on florisil with 10% ether:benzene (10 ml); 20% ether:benzene (10 ml); 30% ether:benzene (10 ml); 40% ether:benzene (10 ml); 50% ether: benzene (10 ml). Each of the eluates were analyzed by GC. Both E_1OMe and E_2OMe were eluted in the first (10% ether: benzene) wash. Thus, further separation of E_1OMe and E_2OMe

by TLC or PC was necessary when using EA for quantitation of E_1 and E_2.

The time dependency of the reaction and the dependence on the presence of THAH for formation of the estrogen derivatives was studied. To a series of each of E_1 (50 µg) or E_2 (50 µg) in 6 ml NaOH was added 50 µl THAH and 5 ml $CH_3I/CHCl_2$ as above. The mixture was shaken for 1, 3, 5, or 10 min. The organic and aqueous phases were separated by centrifugation immediately after shaking was stopped, the organic phase was taken and dried under nitrogen and analyzed by GC. E_1OMe and E_2OMe only were detected in the methylene chloride from the first (1 min) extraction, indicating that reaction was complete after only one min. When THAH was omitted from the reaction mixture, no estrogen methyl ethers were recovered from the CH_2Cl_2. Furthermore, when the NaOH from experiments in which THAH was omitted was taken and THAH and CH_3I/CH_2Cl_2 added as above, estrogen methyl ethers were found in the CH_2Cl_2, demonstrating that in the absence of THAH, the underivatized estrogen remained in the aqueous phase.

Mass spectroscopy of the benzene:ether eluate from florisil chromatography of the EA of E_1 (M^+ 270) gave M^+ 284 and a mass spectrum corresponding to that of authentic E_1OMe (M^+ 284, Table 16). Similarly for the EA of E_2-17β or E_2-17α

(M^+ 272). Mass spectra of authentic E_2OMe-17β and E_2OMe-17α (M^+ 286, Table 17) respectively were obtained. These observed mass spectra agree well with the previous data of Okerholm et. al. (1971).

EA of E_3 gave only one product (E_3-AE). This material did not correspond to authentic estriol 3-methyl ether (E_3OMe) by GC (Table 14) or TLC (Table 18). It formed a silylated derivative which did not correspond to silylated E_3OMe. Acetylation gave only one product which migrated faster than the underivatized material in TLC-E (Table 18). The E_3-EA was eluted in the 50% benzene:ether eluate from florisil columns. MS of the derivative gave M^+ 316 corresponding to a dimethoxy substituted compound (Fig.14). EA of E_3 with dimethyl-(d_6)-iodide substituted for the methyl iodide in CH_3I/CH_2Cl_2 with all other conditions identical to those described above gave the dimethyl-(df) derivative (E_3-AE-CD_3 M^+ 322, Fig.15). Silylated derivatives of E_3-AE and E_3-AE-CD_3 were prepared pure by GC, however difficulty in preparing a crystalline sample for MS was encountered and MS of a silylated derivative was not obtained.

Recoveries of the methylated derivatives of E_1, E_2-17β and E_3 were determined in three series of ten extractive alkylations. Estrogen (50 μg) with the appropriate

tracer added was alkylated as described above. The benzene-ether eluates after florisil chromatography of each of the thirty individual extractive alkylations were taken separately, dried under nitrogen and small aliquots (2%) taken for GC. The presence of only E_1OMe, E_2OMe or E_3-AE was confirmed by GC of each sample. The remaining material from each extraction was then counted in 10 ml toluene/PPO/POPOP. From each extraction, the recovery of methylated estrogen was determined as:

$$\frac{\text{dpm in benzene:ether (methyl ether product)}}{\text{dpm added (as starting material)}} \times 100\%$$

Recoveries were calculated to be 98.6 ± 0.9% (S.E.) for methylation of E_1, 97.6 ± 0.9% for methylation of E_2-17β and 96.4 ± 0.8% for methylation of E_3. Crystallization of ^{3}H-E_1OMe in acetone-hexane and ^{3}H-E_2OMe-17β in acetone:water with appropriate carrier indicated that the products were more than 95% pure. Radioactive E_2-17α was not available for recovery studies.

Modification of this mild alkylation procedure allowed for facile methylation of 2-hydroxyestrone, a compound easily oxidized in aqueous base. Standard 2-OHE_1 (10 mg), 2-methoxyestrone (2-OMeE_2) (5 mg), 2,3-dimethoxyestrone (2,3-diOMeE_1) (10 mg), 2-hydroxy-3-methoxyestrone (2-OHE_1-OMe) and 2-hydroxyestrone-2,3-methylene ether (2 mg) were supplied

courtesy of Dr.K.I.H.Williams. Due to the instability of 2-OHE$_1$, caution was necessary to insure purity. In spite of a slight yellow-brown discoloration of the material on receipt, preparation of the TMS derivative and GC showed the material to be essentially 100% pure (Fig.16). Similarly, the purity of 2-OHE$_1$-OMe and 2-OMeE as the TMS derivatives was found to be 100%. 2,3-diOMeE$_1$ gave one peak by GC which was unchanged in the presence of silylating reagents. The standards and derivatives were stored under air at -20°C. The purity of 2-OHE$_1$, 2-OHE$_1$-OMe and 2-OMeE$_1$ (TMS derivatives) stored in benzene:ethanol (9:1) was checked regularly and after two months storage in solvent at a concentration of approximately 1 mg/ml only slight impurity ($<2\%$) was found by GC.

Methylation of 2-OHE$_1$ was as follows. 2-OHE$_1$ (0.05-1.0 mg) was transferred directly to a 15 ml silanized glass test tube with a Teflon-lined screw-cap stopper. 0.5 M CH_3I/CH_2Cl_2 (5 ml) was added, 0.1 M THAH (100 µl) was added and the tube was flushed with argon and shaken gently to mix. 0.1N NaOH (5ml) was carefully layered onto the organic phase so as not to mix with the methylene chloride. The tube was again flushed with argon, capped and shaken vigorously for 2 min. No colour change was observed. The organic and aqueous phases were separated by centrifugation and the organic phase was dried under nitrogen and used for gas

chromatography directly or transferred to a florisil column and developed with methylene chloride (5 ml). The derivatives were eluted with 10 ml ether followed by 10 ml ethyl acetate. The eluates were collected separately and dried under nitrogen. GC analysis of the organic phase directly showed material with a retention time corresponding to that of authentic 2,3-di0MeE_1 (Table 19, Fig.17). This material was eluted with ether and ethyl acetate during florisil chromatography of the reaction mixture and analyzed by MS, to give a mass spectrum identical to that of authentic 2,3-di0MeE_1 (Fig.18) and in good agreement with the previous data of Hoppen and Siekmann (1974). Similarly, EA of 2-0MeE_1 and 2-0HE_1-0Me gave 2,3-di0MeE_1 (Table 19). Comparison of the relative peak areas from the GC data for the starting material (2-0HE_1 as the TMS derivative) for the methylation of 2-0HE_1 with that of the product, 2,3-di0MeE_1 suggested a recovery in the organic extract of 52-60% (Fig.17). However, since proper internal standards were not included in the GC peak height analysis this estimate can only be approximate. Radiolabelled 2-0HE_1 and its methylated derivatives were not available from any source and it was not within the scope of the present work to attempt synthesis. Estimation of recovery by UV absorption was considered. The absorption maxima of 2,3-di0MeE_1 was determined to be 234 mu in methanol (Fig.19) and a standard curve for quantitation of

2,3-di0MeE_1 in methanol was constructed (Fig.20). A commercial supply of 2-0HE_1 was necessary at this time but no source willing to supply the material in a pure form could be found and further recovery studies were abandoned.

The methylation of 2-0HE_1 was possible only after dissolving the compound first in CH_3I/CH_2Cl_2 with THAH present, before the addition of NaOH. If 0.1 N NaOH was added prior to methylating agents or if the THAH was not mixed into the organic phase prior to the addition of NaOH, rapid oxidation as indicated by the appearance of yellowing of the mixture and the appearance of at least 7 products by GC occurred.

FIGURE 12: Gas chromatography of estrone and estradiol-17β extractive alkylation

A standard E_1 and E_1OMe

B EA of E_1

C EA of $E_2$17β

Varian 2100 FID GC with 6" x 1/4" (I.D.) glass column; 1.5% SE-52 on Chromosorb 750, 80/100; injection 305°C; FID, 305°C; oven, 235°C; N_2 carrier gas 40 ml/min; 0.5 µg; attenuation 16 x 10^{-11}

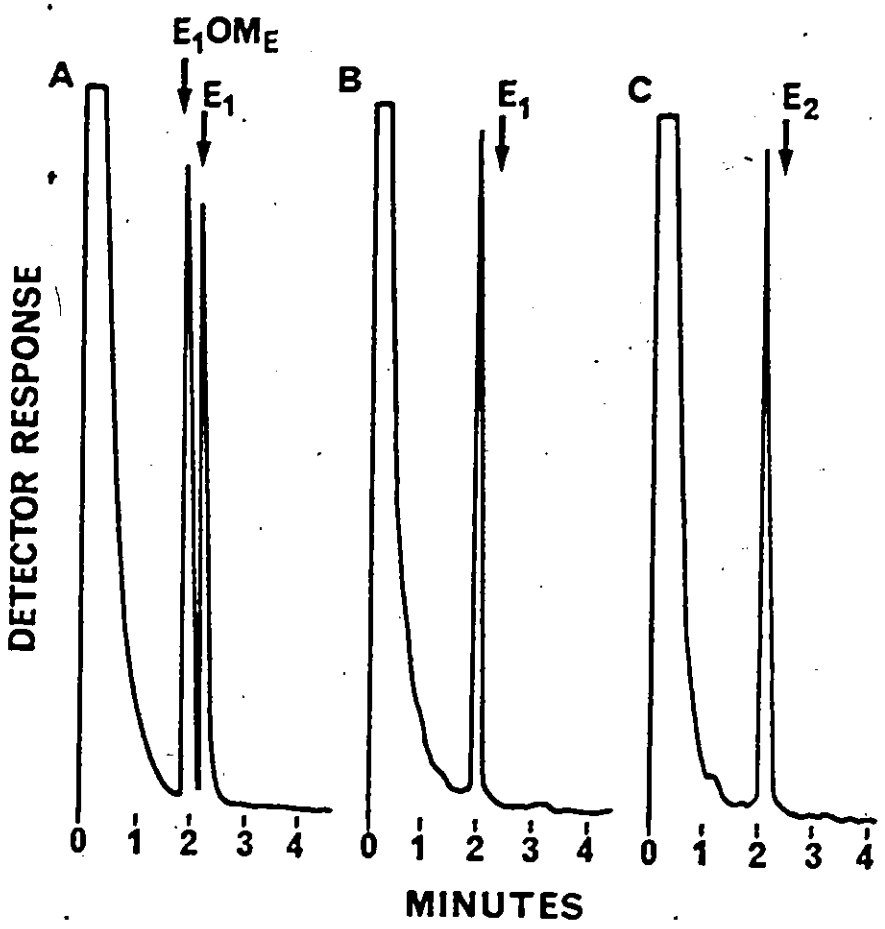

E_1OM_E
A
E_1
B
E_1
C
E_2
DETECTOR RESPONSE
0 1 2 3 4
0 1 2 3 4
0 1 2 3 4
MINUTES

FIGURE 12

FIGURE 13: Column chromatography of ^{3}H-estrone and ^{3}H-estradiol-17β extractive alkylation

Methylene chloride extract from EA of ^{3}H-E_1 and ^{3}H-E_2 on 18 x 0.6 cm florisil columns. Eluting solvent, benzene: ether (1:1).

Each point represents radioactivity in one fraction (20 drops)

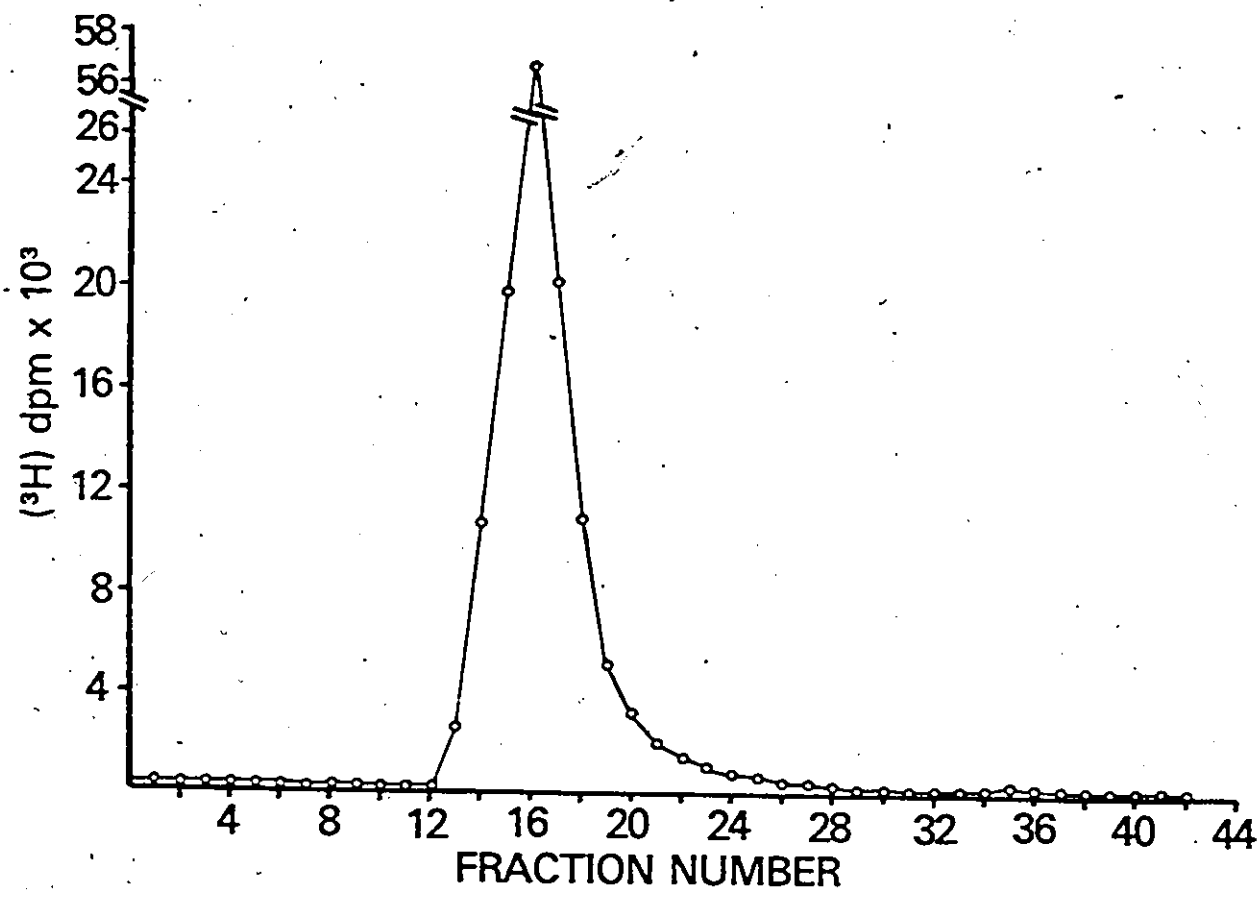

58
56
26
24
20
16
12
8
4
(³H) dpm x 10³
4
8
12
16
20
24
28
32
36
40
44
FRACTION NUMBER

FIGURE 13

FIGURE 14: Mass spectrum of estriol extractive alkylation: non-deuterated derivative

Product of EA with CH_3I
Spectrum obtained on
Consolidated Electrodynamics
Model 21-110B Mass Spectrometer,
ion source 150°C

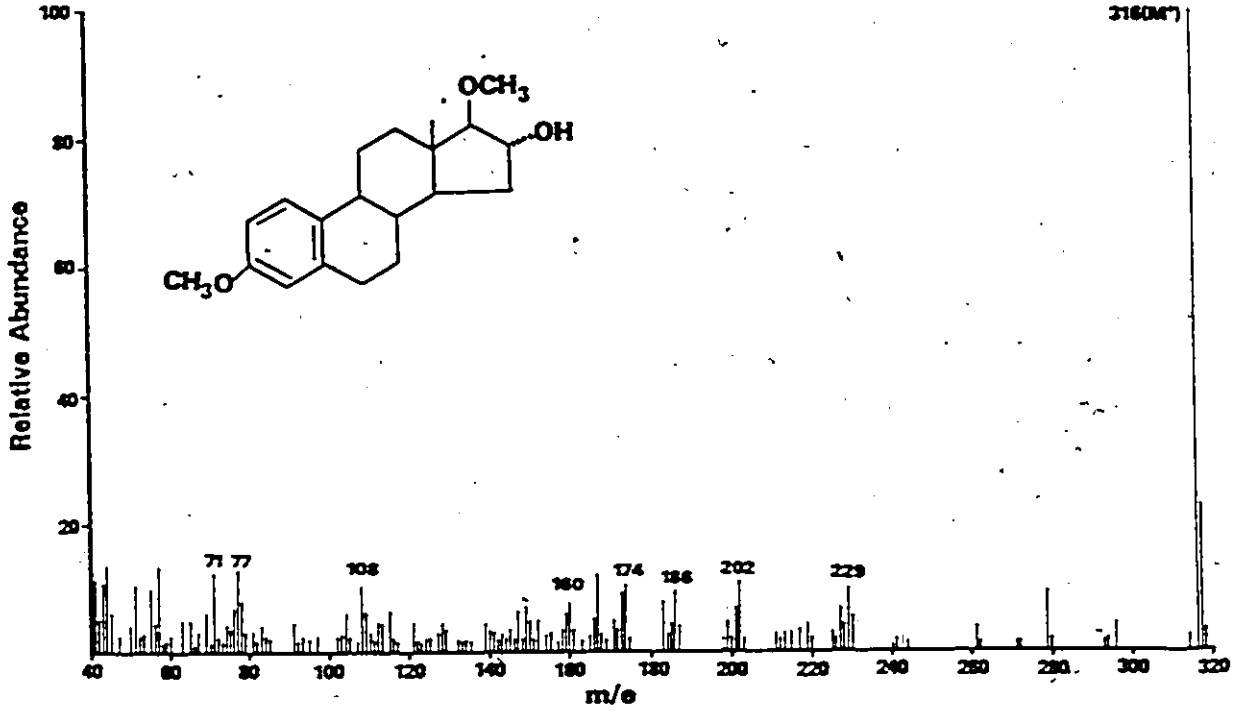

OCH3
OH
CH3O
Relative Abundance
m/e
316(M+)
71
77
108
160
174
186
202
229

FIGURE 14

FIGURE 15: Mass spectrum of estriol alkylation: deuterated derivative

Product of EA with CD_3I
Spectrum obtained on
Consolidated Electrodynamics
Model 21-110B Mass Spectrometer,
ion source 150°C

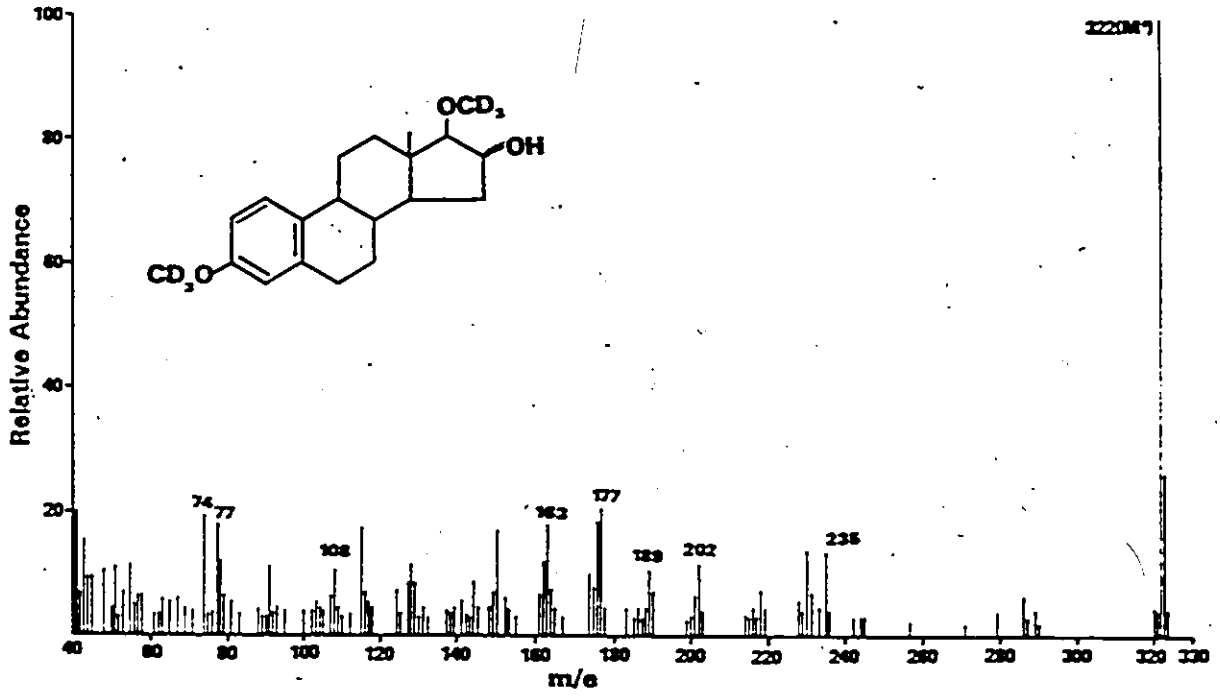

OCD3
OH
CD3O
Relative Abundance
m/e
74
77
108
163
177
189
202
235
320
100
80
60
40
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
330

FIGURE 15

FIGURE 16: Gas chromatography of catechol estrogen standards

(a) 1.0 µg 2,3-diTMS-E_1

(b) 50 ng 2,3-diTMS-E_1

(c) repeat (b)

Varian 2100 FID GC with 6" x 1/4" (I.D.) glass column: 3% OV-17 on Chromosorb W(HP) 80/100; oven 270°C; attenuation 16×10^{-11}

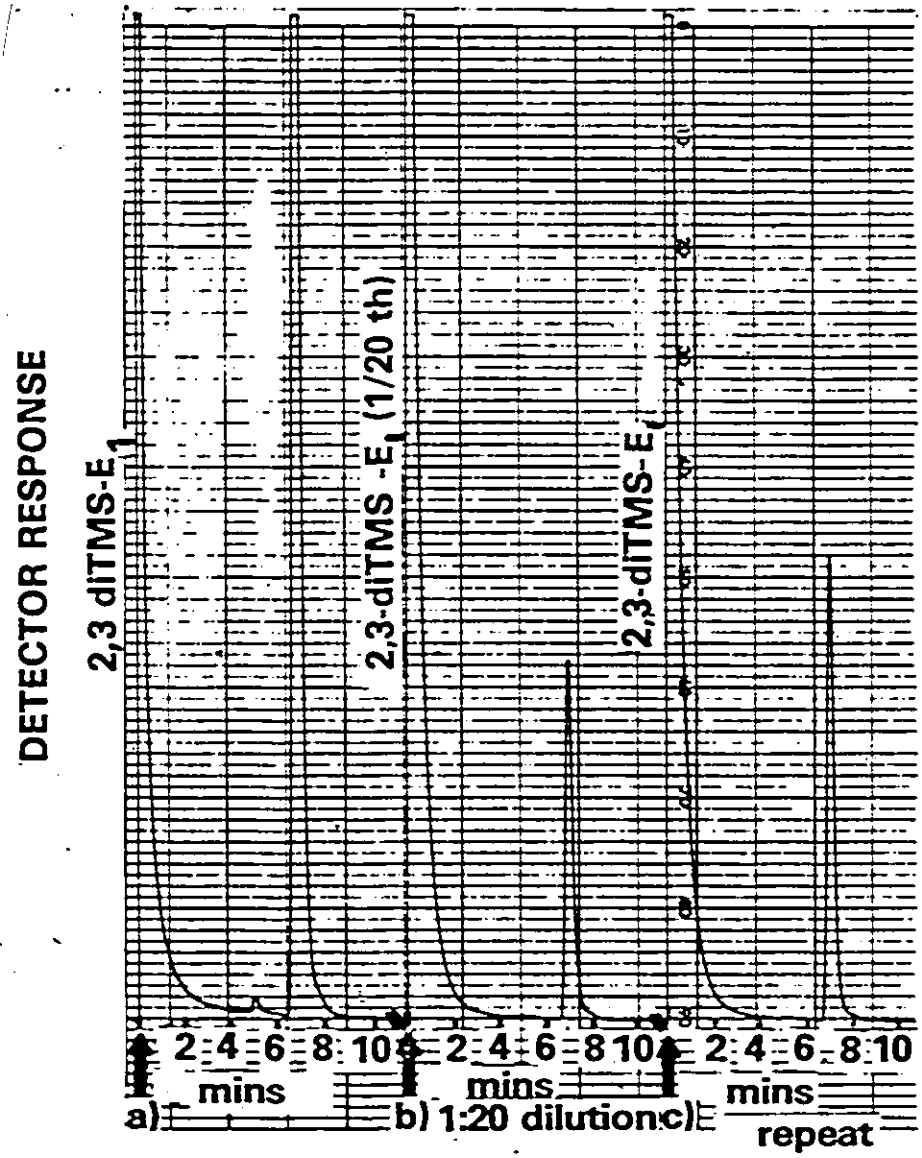

DETECTOR RESPONSE
2,3 diTMS-E1
2,3-diTMS -E1 (1/20 th)
2,3-diTMS- E1
2 4 6 8 10
mins
a)
2 4 6 8 10
mins
b) 1:20 dilution
2 4 6 8 10
mins
repeat
c)

FIGURE 16

FIGURE 17: Gas chromatography of catechol estrogen extractive alkylation

(a) I standard 2,3-diOMeE_1

(b) repeat

(c) blank, ether:ethyl acetate eluate from florisil column

(d) EA of 2-OMeE_1

I - blank peak

II - 2,3-diOMeE_1

(e) EA of 2-OHE_1-OMe

I - blank peak

II - 2,3-diOMeE_1

(f) EA of 2-OHE_1

I - blank peak

II - 2,3-diOMeE_1

Varian 2100 FID GC with 6' x 1/4" (I.D.) glass column; 3% OV-17 on Chromosorb W(HP) 80/100; oven 270°C; attenuation 16 x 10^{-10}

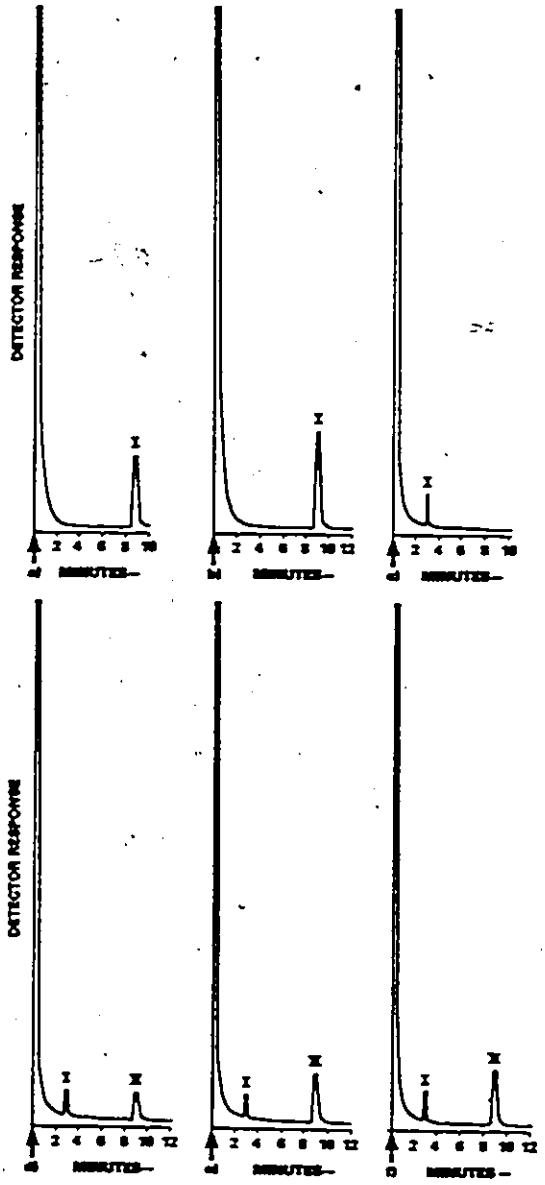

DETECTOR RESPONSE
I
II
MINUTES
DETECTOR RESPONSE
I
II
MINUTES

FIGURE 17

FIGURE 18: Mass spectrum of 2-hydroxy-estrone extractive alkylation

Product of EA of 2-OHE_1

Spectrum obtained on
Consolidated Electrodynamics
Model 21-110B Mass Spectrometer,
ion source 200°C

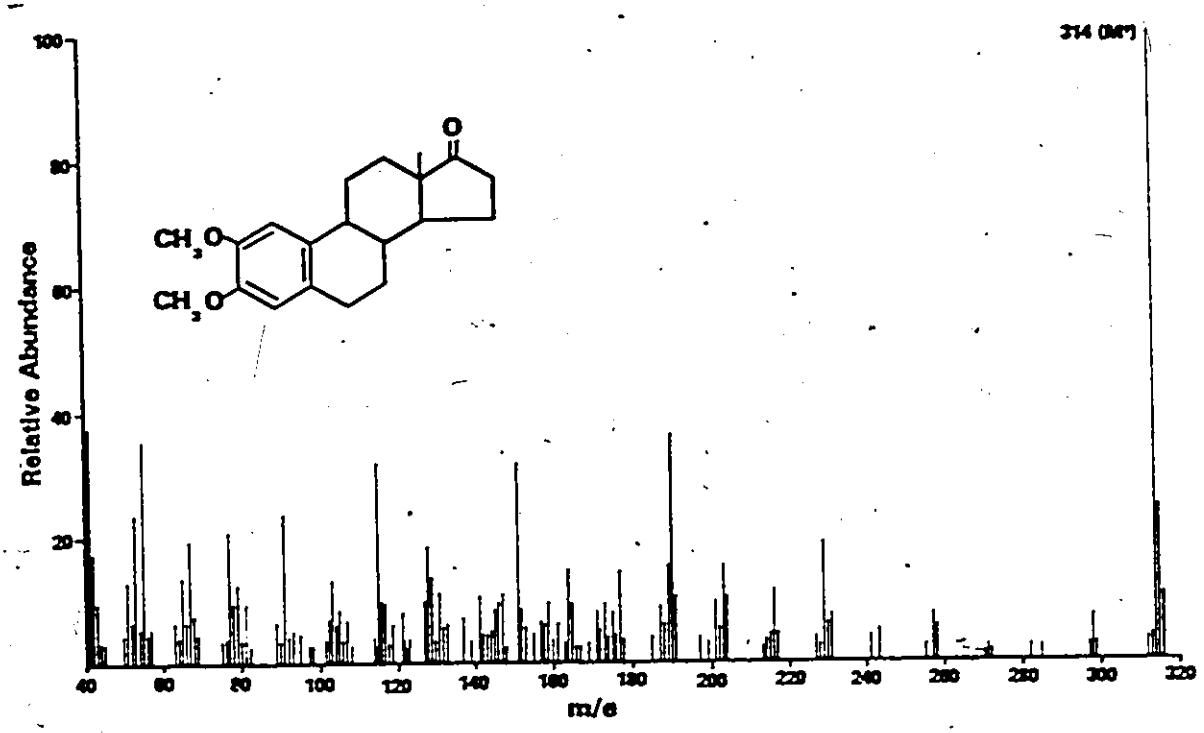


FIGURE 18

FIGURE 19: Absorbance spectrum of 2,3-dimethoxyestrone in methanol

o - 2,3-dimethoxyestrone standard in methanol (100 µg/ml)

▲ - methanol blank

pathlength 1 cm

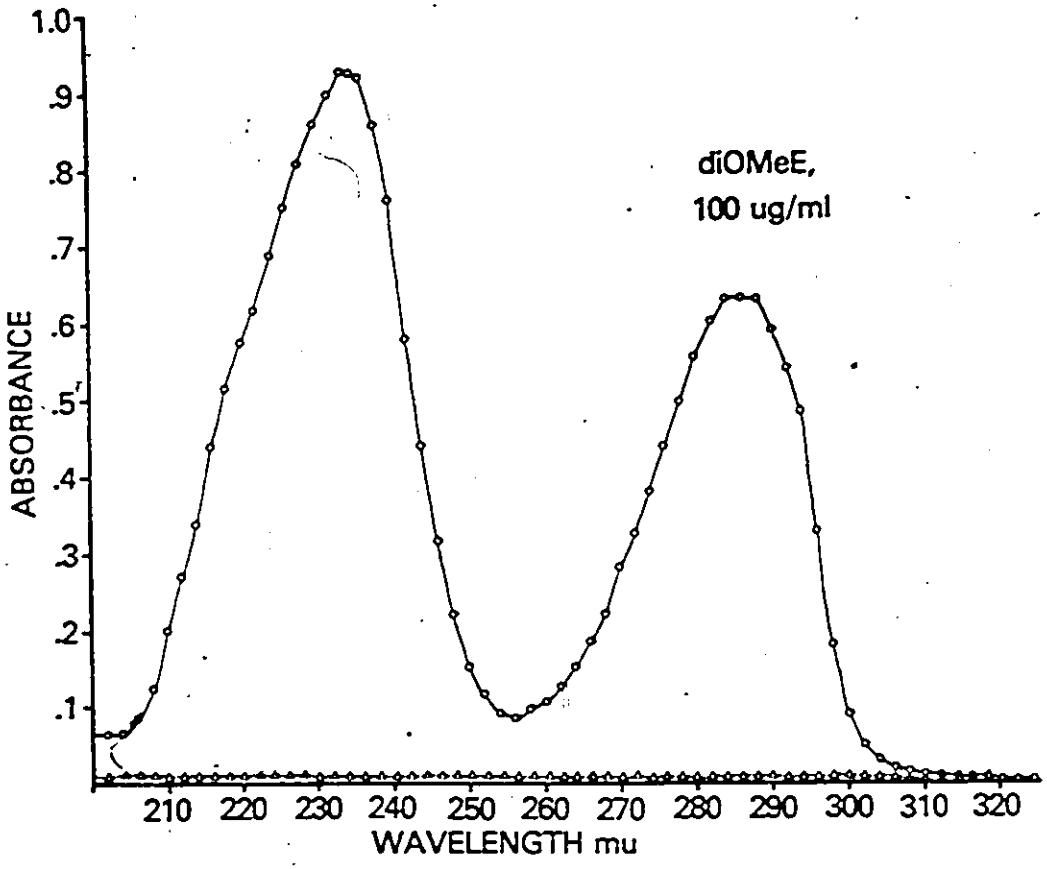

1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
ABSORBANCE
diOMeE,
100 ug/ml
210 220 230 240 250 260 270 280 290 300 310 320
WAVELENGTH mu

FIGURE 19

FIGURE 20: Absorbance of 2,3-dimethoxyestrone in methanol of 234 mμ

o - Each point represents a single determination

Pathlength 1 cm

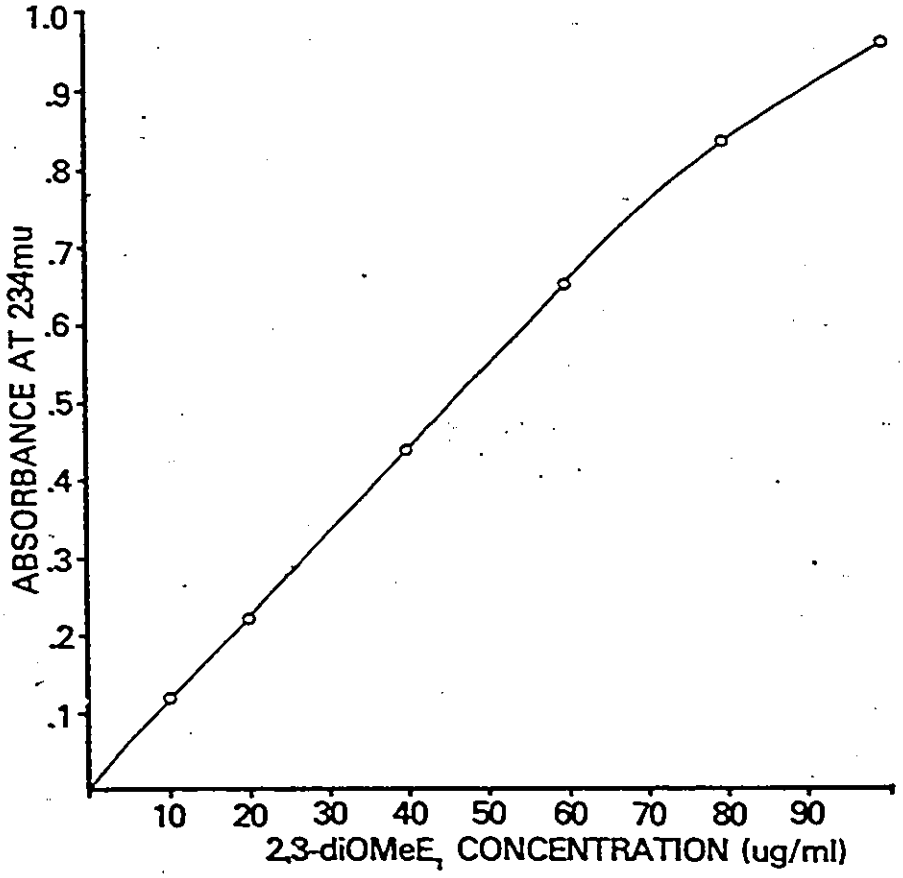

1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
ABSORBANCE AT 234mu
10
20
30
40
50
60
70
80
90
2,3-diOMeE, CONCENTRATION (ug/ml)

FIGURE 20

Table 14. Gas chromatography of estrogen methyl ethers*

standards	retention time≠
estrone	0.815
estradiol-17α	0.741
estradiol-17β	0.722
estriol	1.722
3-methoxyestrone	0.629
3-methoxyestrone-17α	0.669
3-methoxyestrone-17β	0.629
3-methoxyestriol	1.236
extracts≠	
estrone-EA	0.629
estradiol-17α-EA	0.670
estradiol-17β-EA	0.629
estriol-EA	0.964

* Varian 2100, 6 ft x 1/4 in 3% OV-17 Chromosorb WHP 80/100 mesh 270°C

≠ relative to testosterone acetate which has a retention time of 9.40 min.

≠ organic phase from extractive alkylation:retention times unchanged after trimethylsilylation of extracts

Table 15. Thin-layer and paper chromatography of estrogen extractive alkylations.

standards	R_f in solvent system		
	TLC-C	TLC-D	Tol/PE
estrone	0.43	0.13	0.03
estradiol-17β	0.26	0.04	0.001
3-methoxyestrone	0.58	0.33	0.83
3-methoxyestrone-17β	0.35	0.13	0.62
extracts*			
estrone-EA	0.57	0.34	0.83
estradiol-17β-EA	0.36	0.14	0.61

* organic phase from extractive alkylation

Table 16. Gas chromatography-mass spectroscopy of estrogen methyl ethers.
1. Estrone and estrone-3-methyl ether.

E_1		E_1 - AE*	
rel. abun.	mass	rel. abun.	mass
11.2%	73	13.9	55
13.1%	77	11.3	71
13.2%	79	14.2	75
17.3%	91	10.4	77
10.1%	97	12.8	91
10.6%	105	29.3	115
14.0%	107	15.3	131
16.5%	115	20.5	145
11.1%	116	69.4	147
17.9%	120	38.8	159
12.6%	127	75.3	160
14.8%	128	20.3	161
20.8%	131	22.5	171
16.9%	133	33.9	173
18.1%	144	46.3	186
27.6%	145	17.9	197
55.0%	146	71.8	199
13.0%	147	73.3	221
24.0%	157	17.5	222
24.1%	158	13.2	267
26.0%	159	11.3	268
25.8%	160	34.8	281
14.8%	170	13.8	283
15.0%	171	100.0	284
36.3%	172	20.5	285
16.8%	173		
51.6%	185		
18.5%	186		
10.6%	207		
13.7%	211		
25.9%	213		
17.6%	214		
15.8%	226		
15.3%	242		
100.0%	271		
21.7%	272		

* benzene:ether eluate from extractive alkylation of estrone GC-MS conditions as described in 'General Methods Varian MAT CH7 Mass Spectrometer

Table 17. Gas chromatography-mass spectroscopy of estrogen methyl ethers. 2. Estradiol-17β and estradiol-17β-3-methyl ether.

E_2-17β		E_2-17β-OMe		E_2-AE*	
rel.abun.	mass	rel.abun.	mass	rel.abun.	mass
11.4	77	10.6	77	10.5	133
21.6	91	14.6	79	20.0	134
10.3	93	13.7	91	23.2	135
11.5	95	10.6	105	13.9	145
24.1	115	17.1	107	85.1	147
15.2	121	10.2	108	11.3	148
18.3	128	14.3	115	15.5	149
15.5	129	13.5	116	48.0	159
16.3	134	15.7	120	75.0	160
15.5	135	28.6	131	28.8	171
10.1	141	21.1	133	62.1	173
13.6	144	14.4	141	57.0	174
13.1	145	17.2	144	71.2	186
80.1	147	23.4	145	10.6	193
16.7	148	47.8	146	14.3	195
23.3	158	13.5	147	22.1	197
30.1	159	20.2	157	33.5	199
41.5	160	15.1	158	37.0	207
12.1	161	27.2	159	87.0	221
23.2	171	24.8	160	43.4	227
13.2	172	13.1	170	16.9	267
40.4	173	14.2	171	13.4	269
35.1	174	40.2	172	67.4	281
14.7	185	11.7	173	100.0	286
64.4	186	48.4	185	27.7	315
16.7	187	26.0	186	29.5	316
10.8	197	16.7	207	21.5	324
30.3	199	28.5	213	61.3	342
28.7	200	17.0	214	42.2	355
10.3	221	15.1	226	18.4	356
28.0	227	11.5	242	40.5	369
100.0	286	100.0	270	41.0	394
18.6	287	22.8	271		
14.4	315				

* benzene:ether eluate from extractive alkylation of estradiol-17β
Varian MAT CH7 Mass Spectrometer

Table 18. Thin-layer chromatography of estriol extractive alkylation

standards	Rf in solvent system			
	C	E	F	G
estriol	0.03	0.20	0.24	0.14
3-methoxyestriol	0.04	0.28	0.26	0.26
extracts*				
estriol-EA	0.22	0.48	0.45	0.56
estriol-EA-ac+	n.d.	0.63	n.d.	n.d.

*benzene:ether eluate from EA of E_3
+acetate derivative of estriol EA
n.d. - not done; EA - extractive alkylation

Table 19. Gas chromatography of catechol estrogen and methylated derivatives

standards	retention time[/]
2-hydroxyestrone (TMS)	0.849
2-methoxyestrone (TMS)	0.946
2-hydroxy-3-methoxyestrone (TMS)	0.946
2,3-dimethoxyestrone	1.092
extracts*	
2-hydroxyestrone-EA	1.092
2-methoxyestrone-EA	1.092
2-hydroxy-3-methoxyestrone-EA	1.092

* organic phase from extractive alkylation: retention times unchanges after trimethylsilylation of extracts

/ relative to testosterone acetate which has a retention time of 9.4 min.

(f) Hypothalamus and Cortex Incubations with 4-^{14}C-Testosterone: 29 day old Normal Mice

A total of 14 incubations of hypothalamus and cortex tissues were performed using 32 mice. For the first four incubations (10) BALB/c♂ and (10) BALB/♀ mice (29 days old) were killed by cervical dislocation and decapitated. Hypothalamus and cortex tissues were obtained as follows. Using a sharp pair of small scissors, the skin on the head was cut down the midline, exposing the skull cap. The skull cap was then split, again down the midline, thereby exposing the brain. The brain was carefully lifted out of the brain cavity and the pituitary stalk was severed. Using a sharp scalpel, a block of tissue approximately 2 mm cubed, bounded anteriorly by the optic chiasm, posteriorly by the mammilary bodies and on both remaining sides by the limbic areas, was cut and placed in isotonic saline on ice. The hypothalamic blocks thus obtained from 2-10 mice were combined, minced finely with scissors, blotted dry and weighed. Cortex slices were also taken and minced in a similar manner.

The hypothalamus and cortex minces were then transferred to 10 ml Erlenmeyer flasks containing (4-^{14}C-) T (5μCi), nicotinamide adenine dinucleotide phosphate (NADP, 10 μmole), adenosine triphosphate (ATP; 10 μmole), glucose-6-phosphate (G-6-P, 60 μmole) and glucose-6-phosphate

dehydrogenase (G-6-PD, 20 units) in a total volume of 1.0 ml Hank's balanced salt solution, pH 7.4. A control incubation containing no tissue was also prepared. All flasks were tightly capped with Vacutainer rubber stoppers, purged with 95% O_2/5% CO_2 and incubated in a shaking water bath at 100 oscillations/min for 3 hr at 37°C. Incubations were terminated by quick freezing and stored at -78°C until extraction.

To each incubation flask, 50 µg each of E_1, E_2 - 17β and E_3 in 0.1 ml ethanol each and tracer amounts (20,000 dpm each) of ^{3}H-E_1, ^{3}H-E_2-17β and ^{3}H-E_3 were added for recovery. The tissue and medium were then extracted with 3 x 10 ml ether using a Burrell mechanical shaker set at speed 7 for 10 min on each extraction. The aqueous and organic layers were separated by centrifugation at 3500 rpm for 10 min between each extraction. The combined organic extracts were dried under nitrogen. Toluene (3 ml) was added to the dried extract and extracted with 1 N sodium hydroxide (2 x 3 ml) using the Burrell shaker for 10 min on each extraction. The combined sodium hydroxide extracts (6 ml) were backwashed with toluene (5 ml) and the sodium hydroxide was used for extractive alkylation. EA was carried out as described in (e) and by Daley et. al. (1976). Briefly, to the sodium hydroxide extract (6 ml)

0.1 M THAH (100 µl) and 0.5 M methyl iodide in methylene chloride (5 ml) was added. The mixture was shaken 10 min on the mechanical shaker and centrifuged 10 min at 3500 rpm to separate the phases. The NaOH was discarded and the CH_2Cl_2 was transferred to a florisil column and eluted with 10 ml benzene:ether (1:1). The benzene:ether eluate was collected and dried under nitrogen. Methanol (100 µl) was added and an aliquot (1 µl) was taken and analyzed by GC to confirm the presence of the estrogen methyl ethers and completion of the methylation reaction. (Fig.21). An additional 4 µl was taken and transferred to counting vials, dried and counted in 10 ml toluene/PPO/ POPOP.

The remaining extract was dried under nitrogen and transferred in 3 x 10 µl methanol to a 20 x 20 cm plastic sheet of silica gel F-254, previously washed in TLC-A. The plates were developed in TLC-C at room temp. This system was previously found to separate E_1OMe ether, E_2OMe-17β, E_2OMe-17α, E_3-diOMe, E_1, E_2-17α, E_2-17β and E_3. Unlabelled standards were run on each thin-layer plate for comparison.

After development, the sheets were dried in air and autoradiographed for 7 days using Kodak RP-14 Medical

X-ray film. Cold standards were visualized by spraying with 3.5% PMA and heating at 80°C for 5 min.

Areas of radioactivity corresponding chromatographically to ^{14}C-E_1OMe (R_f 0.51), ^{14}C-E_2OMe-17β (R_f 0.32) and unidentified radioactivity (R_f 0.37 and 0.26) (Fig. 22) were scraped from the plate and aspirated into a Pasteur pipette packed with 1 cm methanol-washed glass wool. The silica gel was then washed with 5 ml warm methanol and the eluate was collected and dried under N_2. Recoveries by this method were determined in two separate series of 10 elutions of ^{3}H-E_1OMe (50 µg; 20,000 dpm) and ^{3}H-E_2OMe-17β (50 µg; 20,000 dpm) spotted on silica gel. The recoveries of 10 determinations of each steroid were 82.3 ± 1.9 and 80.4 ± 2.0 (mean ± S.E.) respectively.

^{14}C-E_1OMe was identified in all four incubations by recrystallization from acetone:hexane with approximately 10 mg carrier. Similarly approximately 10 mg cold E_2OMe-17β was used to recrystallize each of the eluates corresponding to E_2OMe-17α (Table 20).

The appearance of a spot (R_f 0.37) close to E_2OMe-17β (R_f 0.32) was unexpected. Separation was poor by TLC-C. In a separate chromatographic run in TLC-C, the R_f's of E_2OMe-17β (0.32), E_2OMe-17α (0.37) and 2,3-diOMeE_1

(0.32) were determined. Thus it is possible that the second spot of R_f between that of E_1OMe and E_2OMe-17β may have been formed from 2-OMeE_1 or 2-OHE_1OMe. Formation of 2OH-E_1 and subsequent dimethylation by the AE procedure would have been unlikely, since 2-OHE_1 would have been destroyed by the addition of 1N NaOH (Daley et. al., 1976).

From the average of the specific activities in the crystals in the n and n-1 crystallizations the percent conversion was expressed as a percentage of the starting material (5 μCi 4-^{14}C-T) per 100 mg tissue (wet wt) in 3 hours. The results are shown in Table 21.

(g) Hypothalamus and Cortex Incubations with 4-^{14}C-Testosterone: 30 day old Tfm (o^+) and Tfm (o^{hv}) mice.

A total of 6 incubations of hypothalamus and cortex tissues were performed using 12 mice. Two (2) Tfm (o^+) and 10 Tfm (o^{hv}) mice. Hypothalamus and cortex tissues were obtained as described in section (f). Incubations, extraction, work-up and chromatography were as described.

Proof of identification of estrogens as

products of T metabolism was established by (1) GC analysis of the EA residue to confirm presence of estrogen methyl ether derivatives and completion of the EA reaction, as described in (f). (2) TLC and autoradiography of the EA extracts. (3) Crystallization with carrier.

From the TLC autoradiograph (Fig. 23) areas of radioactivity corresponding chromatographically to ^{14}C-E_2OMe-17β (R_f 0.36), ^{14}C-E_1OMe (R_f 0.56) and one unidentified product (R_f 0.21) were found. These zones were scraped from the TLC plates and recovered as described in (f). These spots were not present in a control incubation without tissue. The results are shown in Table 22. ^{14}C-E_2OMe-17β was identified in all incubations by recrystallization and a small amount of ^{14}C-E_1OMe was identified in the Tfm (o^+) hypothalamus incubation. The small amounts of radioactivity (< 400 cpm) found in the other E_1OMe eluates were too low for any ^{14}C-E_1OMe to be identified by recrystallization.

FIGURE 21: Gas chromatography of extractive alkylation of BALB/c hypothalamus and cortex incubations with ^{14}C-testosterone

(a) - (e) methylene chloride eluates

(a) hypothalamus male

(b) cortex male

(c) hypothalamus female

(d) cortex female

(e) blank (E_1 and E_2 added)

(f) - (j) benzene: ether eluates

(f) hypothalamus male

(g) cortex male

(h) hypothalamus female

(i) cortex female

(j) blank (E_1 and E_2 added)

Varian 2100 FID GC; 4' x 1/8" (I.D.) glass column; 3% OV-17 on Chromosorb W(HP) 80/100; oven 270°C; attenuation 16×10^{-11}

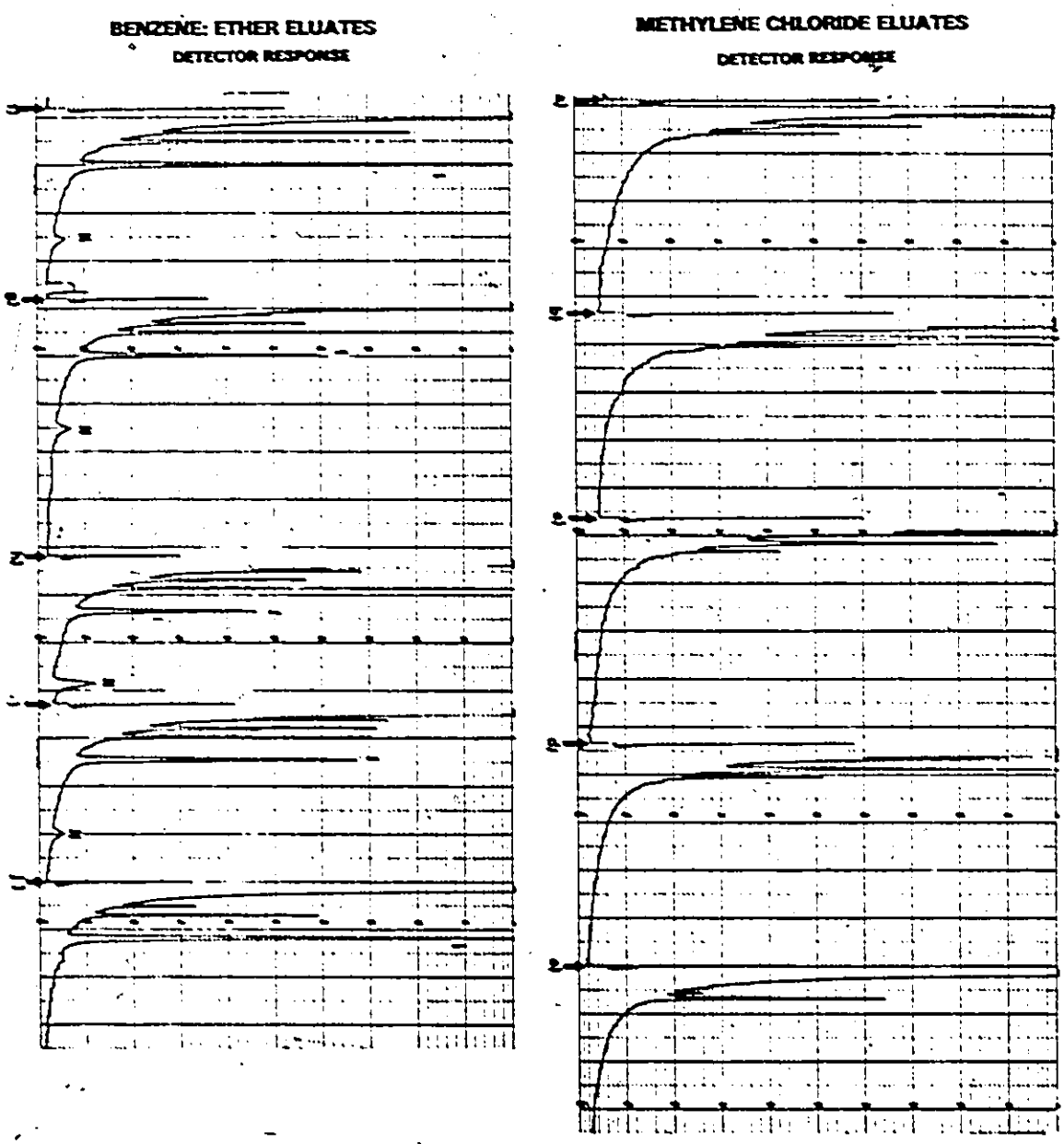

BENZENE: ETHER ELUATES
DETECTOR RESPONSE
METHYLENE CHLORIDE ELUATES
DETECTOR RESPONSE

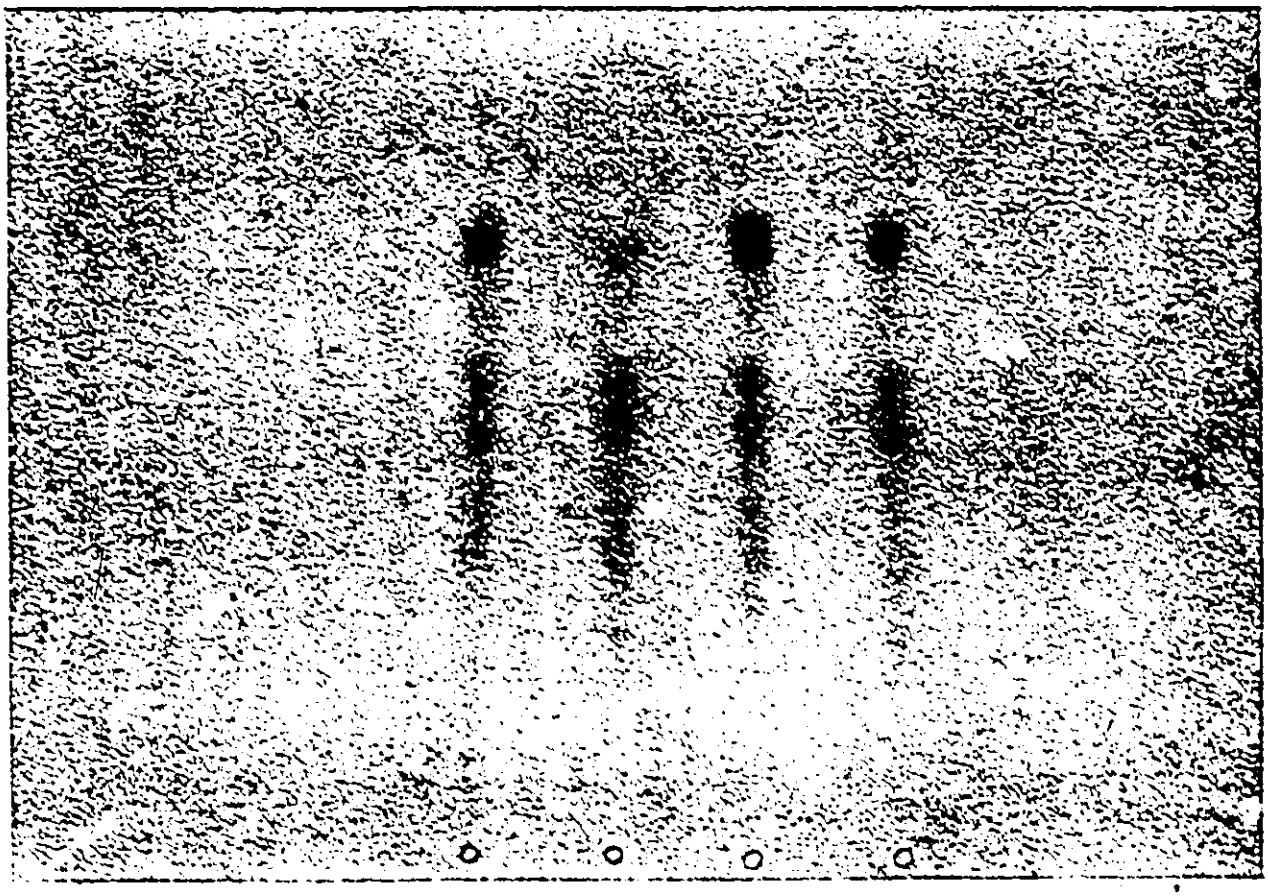
FIGURE 21

FIGURE 22: Thin-layer chromatography of extractive alkylation of BALB/c hypothalamus and cortex incubations with 4-^{14}C-testosterone

Solvent system benzene: ethyl acetate (13:1)

(a) hypothalamus male
(b) cortex male
(c) hypothalamus female
(d) cortex female

Spots corresponding chromatographically, in descending order, to E_1OMe (R_f 0.51), unknown (R_f 0.37), E_2OMe (R_f 0.32), unknown (R_f 0.26) and origin (small spot at bottom).



(a) (b) (c) (d)

FIGURE 22

FIGURE 23: Thin-layer chromatography of extractive alkylation of Tfm (o^+) and Tfm (o^{hv}) hypothalamus and cortex incubations with 4-^{14}C-testosterone

Solvent system benzene:ethyl acetate (13:1)

(1) hypothalamus Tfm (o^+)
(2) cortex Tfm (o^+)
(3) hypothalamus Tfm (o^{hv})
(4) cortex Tfm (o^{hv})

FIGURE 23

Table 20. Recrystallization of ^{14}C-estrone and ^{14}C-estradiol-17β derivatives from thin-layer chromatography of extractive alkylation of phenolic fractions from normal mouse hypothalamus and cortex incubations with 4-^{14}C-testosterone.

	mg tissue wet wt.	E_1OMe mg carrier	E_1OMe n-3	E_1OMe n-2	E_1OMe n-1	E_1OMe n	E_2OMe-17β mg carrier	E_2OMe-17β n-3	E_2OMe-17β n-2	E_2OMe-17β n-1	E_2OMe-17β n
Hypothalamus ♂	122.11	10.430	805.9 / 827.7	731.4 / 821.4	634.9 / 768.6	605.8 / 760.2	12.500	132.5 / 636.5	148.7 / 649.7	130.5 / 658.5	126.7 / 628.9
			(.974)	(.890)	(.826)	(.797)		(.208)	(.229)	(.198)	(.201)
Hypothalamus ♀	126.43	11.621	361.2 / 757.6	357.8 / 791.4	322.6 / 706.8	315.8 / 703.7	10.620	85.6 / 703.8	90.7 / 737.6	72.5 / 721.4	70.8 / 750.6
			(.477)	(.452)	(.456)	(.449)		(.122)	(.123)	(.100)	(.094)
Cortex ♂	97.26	9.785	132.7 / 911.7	128.5 / 890.8	121.0 / 880.4	120.6 / 882.3	9.755	57.2 / 830.0	55.4 / 845.7	49.7 / 821.2	45.8 / 815.6
			(.146)	(.144)	(.137)	(.137)		(.069)	(.066)	(.067)	(.056)
Cortex ♀	135.41	8.290	106.3 / 1061.7	108.5 / 1092.3	94.8 / 1050.8	90.6 / 1041.4	13.050	60.7 / 600.2	62.5 / 590.4	54.0 / 572.7	58.5 / 588.9
			(.100)	(.099)	(.090)	(.087)		(.101)	(.106)	(.094)	(.099)

/ n denotes number of successive crystallizations (4). Upper figure denotes ^{3}H-cpm/mg, lower figure denotes ^{14}C-cpm/mg. Numbers in parentheses are ratios (^{3}H/^{14}C) for each crystallization

Table 21. Aromatization of 4-^{14}C-testosterone by hypothalamus and cortex incubations of 29 day old BALB/c mice

	% conversion/100 mg tissue/3 hr[/]		
	^{14}C-E_1OMe	^{14}C-E_2OMe-17β	total
Hypothalamus ♂	0.12	0.04	0.16
Hypothalamus ♀	0.09	0.02	0.11
Cortex ♂	0.03	0.01	0.04
Cortex ♀	0.02	0.01	0.03

[/] incubation with 5μCi 4-^{14}C-testosterone (57.5 μCi/ mole) in 1 ml Hanks balanced salt solution, pH 7.4 containing NADP 10 μmole), ATP (10 μmole), G-6-P (60 μmole) and G-6-PD (20 units) under 95% O_2/CO_2 at 37°C for 3 hrs.

Table 22. Aromatization of 4-^{14}C-testosterone by hypothalamus and cortex incubations of 30 day old Tfm(o^+) and Tfm(o^{hv}) mice.

		% conversion/100 mg tissue/3 hr		
		^{14}C-E_1OMe	^{14}C-E_2OMe-17β	total E_1 + E_2
Hypothalamus	Tfm (o^+)	.03	.09	0.12
Hypothalamus	Tfm(o^{hv})	n.d.	.11	.11
Cortex	Tfm(o^+)	n.d.	.06	.06
Cortex	Tfm(o^{hv})	n.d.	.04	.04

DISCUSSION

(a) Liver Incubations with 7α-^{3}H-Testosterone

Studies have shown that a number of androgen-dependent liver responses are absent in the Stanley-Gumbreck Ps rat (Bullock et. al., 1971; Neuhaus and Irwin, 1972; Bardin et. al., 1970). These observations have been taken as indirect evidence to support the belief that testicular feminization is characterized by a genetic defect in the androgen receptor protein (Einarsson, et. al., 1972).

It is known that the metabolism of steroid hormones in the rat liver is subjected to large sexual differences (Schriefers, H., 1967). These differences are due to the "organizational effect" of neonatal testicular androgen on steroid metabolizing enzymes during a critical period of development (DeMoor and Denef, 1968; Kraulis and Clayton, 1968).

Einarsson et. al. (1972) found that the metabolism of A and 4-pregnene-3,20-dione in NADPH-supplemented liver microsomes of the Ps rat was different from the normal male pattern. 5α-reduction was 7-10 times greater in Ps than in normal male rats. There was no

3β-hydroxysteroid oxidoreductase for A in Ps rats, no 20α-hydroxysteroid oxidoreductase activity for 4-pregnene-3,20-dione, about half as much 20β-hydroxysteroid oxidoreductase activity for 4-pregnene-3,20-dione, about half as much 16α-hydroxylase activity for A and the 6β-hydroxylase activity for 4-pregnene-3,20-dione was about half that in Ps rats as in normals.

Species differences exist between the rat and mouse in the metabolism of T in NADPH supplemented hepatic microsomes, with little ring-A reduced metabolites produced in the mouse (Jagarinec et. al., 1967). In this study, small amounts of the 5α-reduced metabolites DHT and AOR_2 were identified in incubations of mouse liver homogenates (Table 5). The major metabolite of T, identified and quantitated by crystallization was A (Table 7). However, the pattern of T metabolism in BALB/c , Tfm (o^+) and Tfm (o^{hv}) mice incubations was different.

The sum of the percentages (of starting material) recovered as T and A for the BALB/c incubations was 61.9%. Thus, assuming equal recoveries, 38.1% of the starting material was converted to polar metabolites (Fig. 4). Similarly the total percentages of T and A for the Tfm (o^+) and Tfm (o^{hv}) incubations were 53.6% and 44.2% respectively.

leaving 46.4 and 55.8% of the precursor converted to products other than T, A or the 5α-reduced metabolites. Some of the polar, hydroxylated metabolites of T formed in significant yield in mouse liver incubations have been identified as 7α-hydroxy-testosterone and 6β-hydroxytestosterone (Jagarinec et. al., 1967). However, these metabolites were not looked for in this study.

The formation of A from T in all incubations indicates the presence of 17β-OHSDH, as expected, in BALB/c liver and in both Tfm (o^+) and Tfm (o^{hv}) livers. Comparison of the percentage conversion of T to A and the A/T ratios (Table 8) suggests that of the three genotypes studied, the activity of 17β-OHSDH was lowest in the Tfm (o^{hv}) liver. A summary of these results has been presented elsewhere (Daley et. al., 1976).

(b) Testes Incubations with 7α-^{3}H-Testosterone and 4-^{14}C-Progesterone

The most striking differences between the incubations of 7α-^{3}H-T and 4-^{14}C-P with testes minces from BALB/c, Tfm (o^{+}) and Tfm (o^{hv}) are found in the relative amounts of testosterone formed from progesterone (Table 12).

While 23.3% of the radioactivity added to BALB/c incubations as ^{14}C-P was recovered as unchanged ^{14}C-P, 11% of the substrate was converted to ^{14}C-T. Similarly, in the incubations of Tfm (o^{+}) and Tfm (o^{hv}) testes, 34.3% and 13.3% of the substrate was unchanged while 0.8% and 2.2% respectively was converted to ^{14}C-T. The amounts of ^{14}C-A formed from ^{14}C-P were greatestin the Tfm (o^{+}) incubations, (7.5%); less in the Tfm (o^{hv}) incubations, (1.3%); and least in the BALB/c incubations (0.7%). Together this suggests that the relative activities of the 17-ketoreductase for conversion of A to T arein the order: BALB/c > Tfm (o^{hv}) > Tfm (o^{+}). This conclusion is only correct if the rates of formation of other products from A and T in all the incubations are the same.

In their study of testosterone formation from progesterone in testis of the Stanley-Gumbreck Ps rat,

Aronin et. al. (1974) found that in incubations of minced testis without added NADPH, A accumulated more in the Ps incubations that in the normal incubations. T accumulated more in the normal incubations than Ps. Thus, their results are similar to the present work. Similarly, Aronin et. al. found very low formation of AOH_2 and androsterone. However, when the incubations were repeated in the presence of an NADPH-generating system, AOH_2 was the major product recovered from the normal incubations while androsterone was the major product formed in the Ps incubations. Therefore, while the difference in 17-ketoreductase activity was evident in incubations of the normal, Tfm (o^+) and Tfm (o^{hv}) tissues, without the addition of cofactors, it is possible that there may be other differences which only become apparent under different conditions: for example, various NADPH concentrations. However, in the present study, the effects of NADPH on the metabolism of progesterone or testosterone were not investigated.

(c) Testes Incubations with ^{14}C-Progesterone

Ellis and Berliner (1965) demonstrated that both the Δ^4 and Δ^5 pathways (Fig. 2) are present in mouse testes leading to the production of T from P and 5-pregnenolone.

Tsujimura et. al. (1975) have also shown that in immature mouse testis, incubations of NADPH-supplemented homogenates with progesterone indicate two biosynthetic pathways leading to C_{19} steroids from progesterone, one from progesterone via 17α-hydroxyprogesterone and A to T and a second via 5α-reduced C_{21} steroids to 5α-reduced C_{19} steroids such as androsterone and 5α-AOH_2. In similar incubations of adult mouse testes, very little 5α-reduction of all the Δ^4-3-ketosteroids was found. The major metabolites of P were A and T.

As expected when P was incubated with testes minces of BALB/c♂ mice, the major products could be identified as A and T (Fig. 9, Table 12). A similar pattern of P metabolism was found in incubations of testes minces from the genetic mutant mice +Ta++/+++, sxr♂ and Tfm+(o^{hv}) Blo/+++, sxr ♂ (Fig. 9).

XX mice of the genotype +Ta++/+++, sxr ♂ despite a testis which is only about one-tenth the size of the XY testis and totally devoid of germ cells, show almost complete male development.

According to Drews et. al. (1974), Leydig cells in the +Ta++/+++, sxr ♂ testis must produce enough testosterone to maintain development of the Wolffian duct and urogenital sinus derivatives. This assumption is supported by the results of the present study. Testis minces from +Ta++/+++, sxr ♂ mice produced almost 60% as much T from P as the testis minces from normal XY mice.

The situation of the Tfm+(o^{hv})Blo/+++, sxr ♂ is more complicated. Mice of this genotype are produced by crossing Tfm+(o^{hv})Blo/++(o^{+})+, heterozygotes to XY, sxr/+ males. The autosomal dominant sxr gene (Cattanach et. al., 1971) is transmitted to half of the XX progency. The Tfm+(o^{hv})Blo/+++, sxr ♂ progeny are phenotypic males with small penis, hypospadias and small seminal vesicles and prostates.

According to X-inactivation theory (Lyon, 1961), it follows that in the organs of Tfm+(o^{hv})Blo/+++, sxr ♂ are expected to be in the +/- induced state (Drews et.al.,1974)

However, Drews et. al. (1974) have observed that in Tfm+(o^{hv}) Blo/++(o^{+})$^{+}$ sxr♂ mice there is a strong positive correlation between extreme variegation with regard to Blo of the coat and the near complete absence of male genital tracts. This observation has been taken as evidence that the (o^{hv})allele influences the X-inactivation process and causes the X-chromosome carrying (o^{hv}) to be preferentially activated over the (o^{+})-X-gene (Drews et. al., 1974).

Comparison of the amounts of A and T formed from P in testes incubations of BALB/c♂, +Ta++/+++, sxr♂ and Tfm+(o^{hv})Blo/+++, sxr♂ shows that the relative activity of the 3-ketoreductase appears to be in the order BALB/c♂ > +Ta++/+++, sxr♂ > Tfm+(o^{hv})Blo/+++, sxr♂. According to the above argument it appears that the Tfm+(o^{hv})Blo - gene has an effect on steroid metabolism. The exact mechanism of this effect cannot be determined from the present experiments.

(d) Testes Incubations with 7α-^{3}H-Androstenedione

There have been no previous studies reported in which the metabolism of A in testes incubations of Tfm(o^{+}) and Tfm(o^{hv}) mice was compared. In this study the formation of T was similar in all incubations (Table 13 However, larger amounts of A remaining in the Tfm(o^{+}) incubations (Table 13) and comparison of the radiochromatogram scans (Fig. 10) suggests that in addition to T and very polar metabolites at the origin, four metabolites of A were formed in the Tfm (o^{hv}) incubations that were not present in the Tfm (o^{+}) incubations (Fig. 10).

In testes incubations with ^{3}H-T and ^{14}C-P the radiochromatogram scans of the Tfm (o^{hv}) incubations (Fig. 7) also showed two extra peaks appearing as shoulders to peaks I and VI. The significance of the extra peaks appearing in Tfm (o^{hv}) incubations is difficult to determine since none of these extra metabolites could be identified. Their identity may be of interest in view of the differences which have been described between the Tfm (o^{+}) and Tfm (o^{hv}) strains. For example it is possible that metabolites of T or A may be involved in the altered negative feedback responses to steroids observed in the Tfm (o^{+}) and Tfm (o^{hv}) mice (Kan et. al., 1974).

(e) Extractive Alkylation as a Method for Studying Aromatization

During the course of the present work, a rapid and convenient method was developed for detecting small amounts of estrogens formed from androgens in CNS tissues of the mouse. The method is based on the technique of extractive alkylation (EA) previously reported as a quick and convenient procedure for the preparation of a number of alkylated derivatives of phenols in general chemical synthesis (Brändström and Junggren, 1972; McKillop et. al., 1974) and drug analysis (Ervik and Gustavii, 1974; Lindstrom and Molander, 1974). This is the first application of the technique to steroid chemistry (Daley et. al., 1976).

"EA" or "ion-pair partitioning" is a technique which has recently been a subject of interest in preparative organic chemistry (Brändström and Junngren, 1972). The alkylation reaction involves the formation of an ion-pair between the anion of a compound (i.e. a phenol) present in aqueous base and a large cation such as tetra-hexylammonium present as the hydroxide. The ion-pair thus generated is lipid soluble and easily extracted into a methylene chloride solution of the alkylating agent (methyl iodide). This is followed by rapid irreversible

alkylation to give the phenol ether, with concomitant formation of tetrahexylammonium iodide which remains in the organic phase.

The Brown method (Brown, 1955) is widely used for the preparation of estrogen methyl ethers. Other estrogen methylation reactions have been reported, but these require vacuum sublimation (MacGee and Allen, 1970) or stirring overnight (Abdel-Aziz and Williams, 1969) and are therefore unsuitable for outine work. As described by Brown (1955), estrogens may be methylated using dimethyl sulphate. The reaction requires heating at 37°C for 90 min and is complicated by the destruction of dimethyl sulphate by sodium hydroxide.

The method of Naftolin et. al. (1975) for studying aromatization, utilizing the Brown (1955) methylation is outlined in Fig. 24. A large number of transfer steps are involved and the overall recoveries are low (40-60%; Flores et. al., 1973). Toluene-sodium hydroxide partitioning effectively separates neutral and phenolic compounds (Engel et. al., 1950). The "classical" technique for recovery of the estrogens from sodium hydroxide involves addition of acid, ether extraction, extensive washing of the ether extract and chromatography

(Ainsworth and Ryan, 1966). Further purification to give material suitable for crystallization involves methylation of the estrogens with dimethyl sulphate, work-up of the methylation products, further chromatography and finally, crystallization with carrier.

By comparison, the method used in the present study utilizing the "EA" methylation is shown in Fig. 25. The overall number of steps required to obtain the purified estrogen methyl ethers is low and the resulting recoveries are high (80-85%). A major advantage of the EA procedure is that the extraction of estrogen from aqueous base and direct conversion to the methylated derivatives is carried out in one step. Florisil column chromatography rapidly removes the THAI and the methyl iodide is efficiently and rapidly removed by evaporation to leave the purified estrogen derivatives suitable for chromatography and crystallization.

The methylation of E_1, E_2 and E_3 is rapid and gives only one product in all cases. When combined with florisil column chromatography, tetrahexylammonium iodide is removed and the derivatives are recovered quantitatively. If tetrahexylammonium hydroxide was omitted from the reaction mixture, no derivatives were recovered from the organic extract.

E_1OMe, E_2OMe-17α and E_2OMe-17β were identified by PC, TLC and GC-MS. In the case of E_3, the alkylated derivative did not correspond to estriol-3-monomethyl ether. The derivative was determined to be a dimethoxy substituted compound on the basis of acetate derivative formation and M^+ 316 and 322 respectively in the mass spectra of the non-deuterated and deuterated derivatives.

From steric considerations, methylation of C-16α-OH would be expected to proceed more readily than C-17β-OH methylation, due to the presence of an angular methyl group at C-18. However, by comparison of the mass spectra of the non-deuterated (Fig. 14) and deuterated (Fig. 15) derivatives, the identity of the E_3 derivative can be tentatively established as 3-17β-dimethoxy-estriol.

The major ion fragments in the mass spectra (Fig. 14 (15) at m/e 108(108), 160(163), 174(177), 186(189) 202(202) and 229(235) can be assigned to portions of the A, B, C and D rings following the reaction mechanisms of Okerholm et. al., (1971) and Budzikiewiez et. al., (1964). Similarly, following the interpretation of the mass spectrum of estriol-TMS derivatives as discussed by Okerholm et. al. (1971), expansion of ring D, followed by a

1,3-migration of the C-16 group and bond rupture would give a stable molecular ion. For 3,17β-dimethoxyestriol, this ion would occur at m/e 71. Substitution of deuterium at C-17OH would give m/e 74 (Fig. 26). An m/e shift of 71(74) was consistently observed in mass spectra repeatedly recorded for ion source temp of 120°C, 150°C and 160°C. Conversely, the 3,16α-dimethoxyestriol derivative if formed, would have given a peak at m/e 57 in both the non-deuterated and deuterated derivatives. However this ion does not occur in both spectra.

Further proof of the 3,17β-dimethoxy substitution would require ^{13}C-nuclear magnetic resonance spectroscopy (R.Bell, personal communication), however, it was not considered within the scope of the present work to pursue this further.

EA of E_3 gave the dimethoxy product in quantitative yield. This is in contrast to the dimethyl sulphate procedure, which yields predominantly the 3-monomethyl ether, although 3% of the product was a dimethyl ether product which was thought to be the 3,17-dimethyl ether, but not proven (Mathur and Common, 1967). Thus it appears that under these conditions, methyl iodide is a more potent alkylating agent than

FIGURE 24: "Classical" technique for studying aromatization

Brown (1955) and Naftolin (1975)

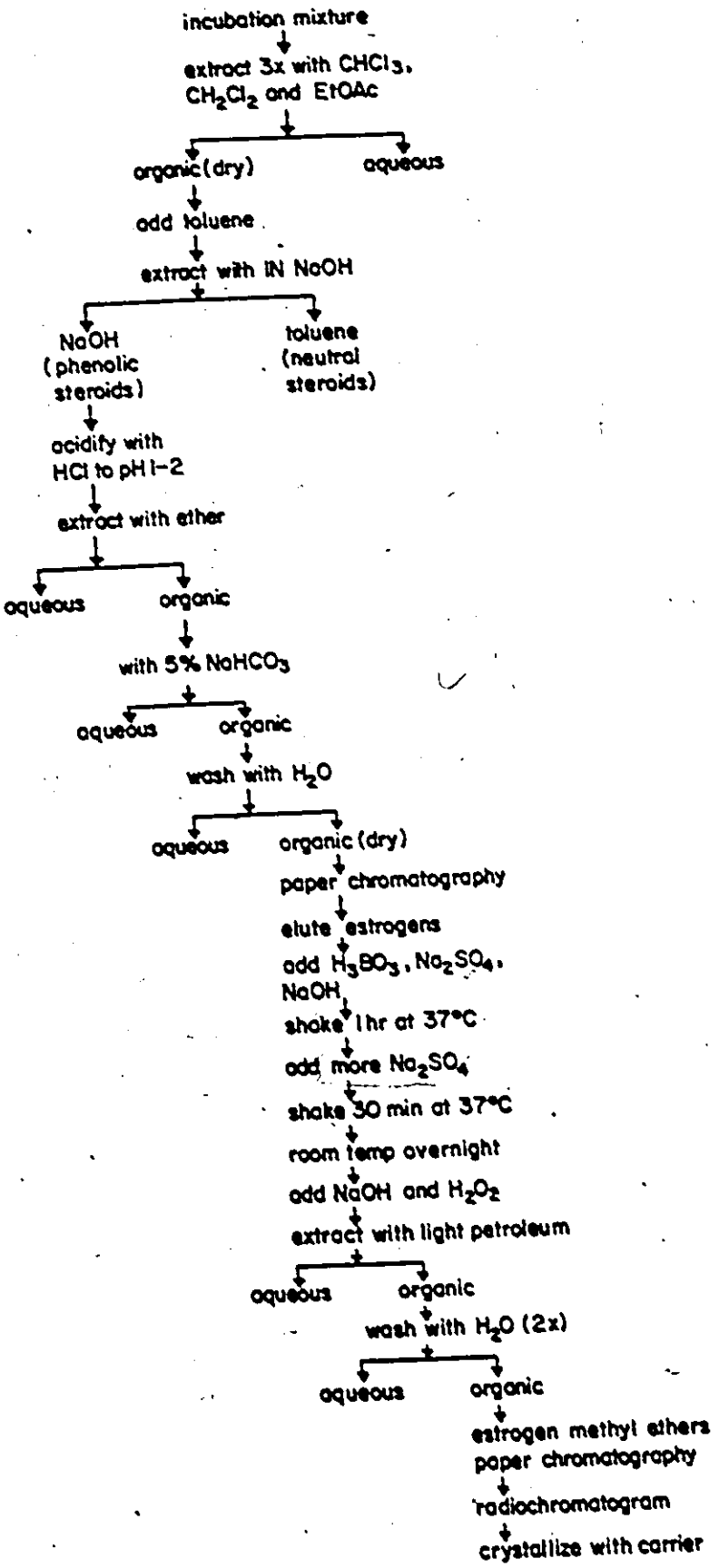

incubation mixture
extract 3x with $CHCl_3$, CH_2Cl_2 and EtOAc
organic (dry)
aqueous
add toluene
extract with IN NaOH
NaOH (phenolic steroids)
toluene (neutral steroids)
acidify with HCl to pH 1-2
extract with ether
aqueous
organic
with 5% $NaHCO_3$
aqueous
organic
wash with H_2O
aqueous
organic (dry)
paper chromatography
elute estrogens
add H_3BO_3, Na_2SO_4, NaOH
shake 1 hr at 37°C
add more Na_2SO_4
shake 30 min at 37°C
room temp overnight
add NaOH and H_2O_2
extract with light petroleum
aqueous
organic
wash with H_2O (2x)
aqueous
organic
estrogen methyl ethers
paper chromatography
radiochromatogram
crystallize with carrier

FIGURE 24

FIGURE 25: Extractive alkylation as a method for studying aromatization

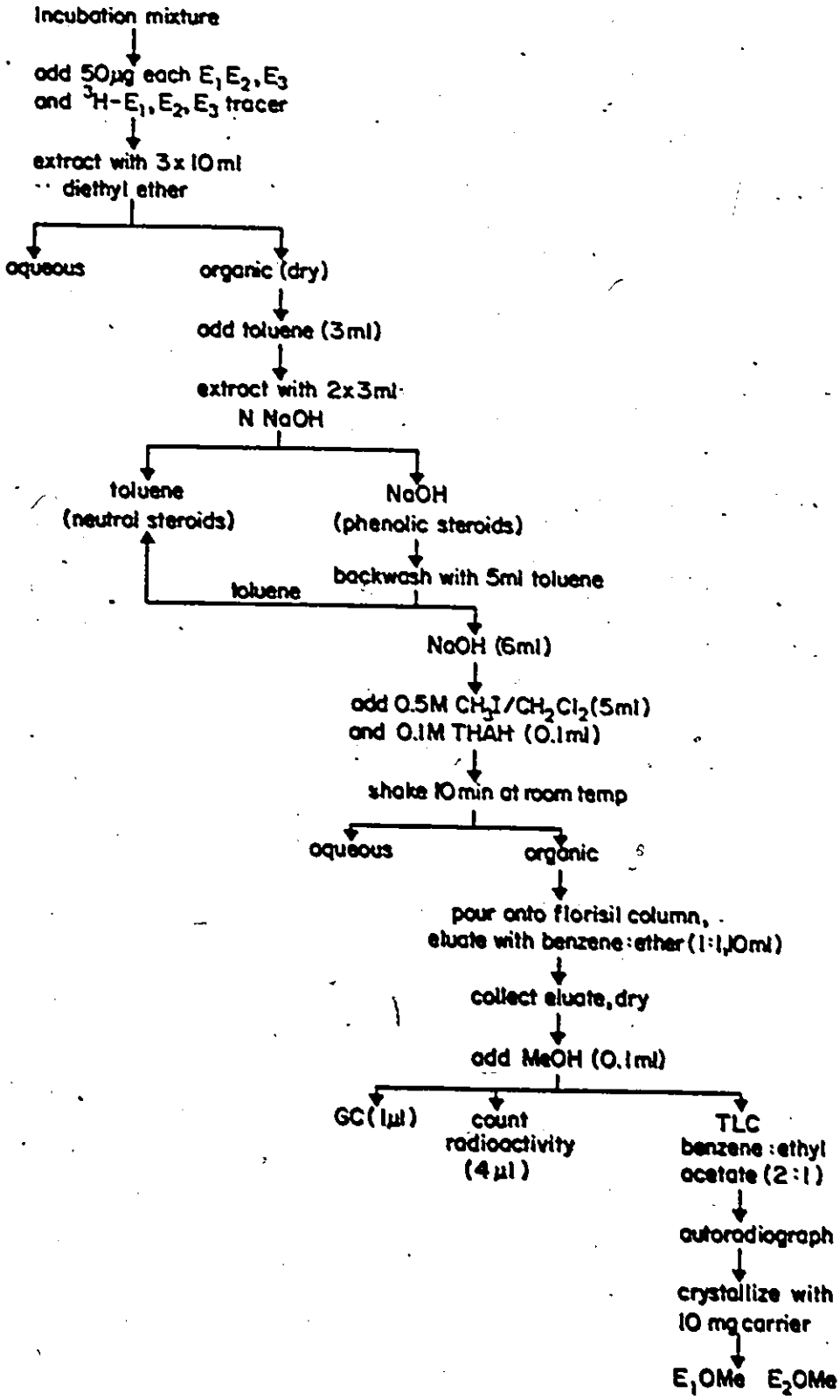

Incubation mixture
add 50µg each E_1, E_2, E_3 and ^3H-E_1, E_2, E_3 tracer
extract with 3 x 10 ml diethyl ether
aqueous
organic (dry)
add toluene (3 ml)
extract with 2 x 3 ml N NaOH
toluene (neutral steroids)
NaOH (phenolic steroids)
backwash with 5 ml toluene
toluene
NaOH (6 ml)
add 0.5 M CH_3I/CH_2Cl_2 (5 ml) and 0.1 M THAH (0.1 ml)
shake 10 min at room temp
aqueous
organic
pour onto florisil column, eluate with benzene : ether (1:1, 10 ml)
collect eluate, dry
add MeOH (0.1 ml)
GC (1 µl)
count radioactivity (4 µl)
TLC benzene : ethyl acetate (2 : 1)
autoradiograph
crystallize with 10 mg carrier
E_1OMe E_2OMe

FIGURE 25

FIGURE 26: Mass fragmentation pattern of 3,17β-dimethoxyestriol-D-ring

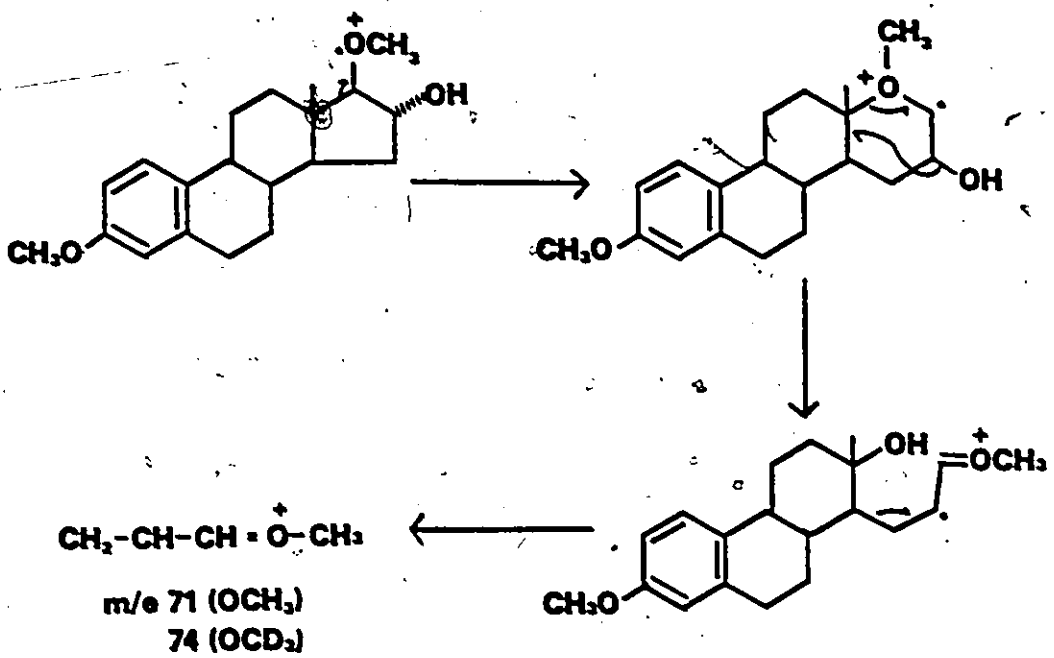

OCH3
OH
CH3O
CH3
O
OH
CH3O
OH
OCH3
CH3O
CH2-CH-CH = O-CH3
m/e 71 (OCH3)
74 (OCD3)

FIGURE 26

dimethyl sulphate, which requires higher temperatures and longer reaction times.

Methylation of catechol estrogens in aqueous alkaline solution has not been previously reported. Due to the instability of the catechol structure to oxidation, formation of 2,3-di0MeE_1 from 2-0HE_1 was unexpected. Since mixing of the catechol with the alkylating agent in the presence of THAH was required before addition of 0.1N NaOH, some "protection" was presumably afforded to the easily oxidized 2,3-diol structure. The formation of 2,3-di0MeE_1 from 2-0HE_1, 2-0MeE_1 and 2-0HE_1-0Me was demonstrated by GC and MS.

Biological formation of catechol estrogens and their transmethylation products has been reported in liver, kidney, placenta, adrenal, ovary and testis (Breuer, et. al., 1961; Troen, 1961; Lucis and Hobkirk, 1963; Lucis, 1965; Axelrod and Goldzieher, 1961, 1962). Catechol estrogen formation in central nervous tissues (Fishman and Norton, 1975) and interaction of catechol estrogens with neurotransmitters at the hypophysial-hypothalamic level (Breuer and Köster, 1974) have also been recently demonstrated. Because an attempt to isolate 2-0HE_1, even by gentle procedures results in very considerable destruction of the compound (Fishman,

1963) assay methods for 2-OHE$_1$ are fraught with difficulties. Methylation of 2-OHE$_1$ by EA to give a stable derivative may be a useful technique for the study of this compound in biological systems. Preparations of tritium, carbon-14 or deuterium labelled compounds for analysis would be possible.

(f) Aromatization of Testosterone in BALB/c , BALB/c , Tfm (o^+) and Tfm (o^{hv}) Hypothalamus and Cortex Incubations

The central neuroendocrine functions of sex steroids are generally believed to involve the triggering and control of the sexual differentiation of the brain, puberty, gonadotrophin synthesis and release, and sexual behaviour. During a critical period (from birth to about 10 days in rats and mice) hormones serve to organize the CNS towards development in postpubertal life of male or female behavioural and neuroendocrinological patterns. Thus, testicular secretions serve to masculinize the neonatal male rodent with respect to the later development of noncyclic LH release and of male behaviour, while the neonatal female, in the absence of these secretions, will express after puberty, the female pattern of behaviour and the cyclic output of LH necessary for

ovulation and the formation of corpora lutea (Gorski, 1971).

Interference during the "critical" stage of maturation may influence the sexual differentiation of the CNS of both males and females and lead to modification of these behavioural and neuroendocrinological patterns. Testosterone administered to female rats or mice during the first weeks after birth causes sterility, polyfollicular ovaries, constant vaginal estrus, an absence of female behaviour and increased male behaviour (Gorski, 1966; Ptaff, 1971). Estradiol-17β is also effective in producing "neonatal masculinization" (Gorski, 1963; Harris and Levine, 1965) similar to that induced by testosterone.

Three theories have been proposed to explain the mechanism of neonatal masculinization. According to one theory, testosterone is the agent responsible for these effects, acting via interaction with specific androgen receptors in the hypothalamus. Androgen binding proteins have been reported in rat and mouse hypothalamus preparations (Jouen et.al., 1971; Mondon et. al., 1974; Naess et al., 1975; Fox, 1975; Attardi et. al., 1976). According to this theory, the absence

of androgen-binding proteins in the hypothalamus would result in the inability of testosterone to induce masculinization. Fox (1975) and Attardi et. al. (1976) have demonstrated greatly reduced androgen binding in the hypothalamus of the Tfm/Y mouse. Thus this animal should be useful for testing this first theory. Although testosterone administration to postpubertal Tfm/Y mice is generally ineffective in invoking any responses (Table 1), the effect of early injections of testosterone has not been studied, to this author's knowledge. However, the level of testosterone in the circulation at the neonatal stage is comparable in Tfm/Y mice to that in normal males (Goldstein and Wilson, 1972). Therefore, absence of the androgen receptor in Tfm/Y mice is consistent with the failure of male development to occur. A further observation implicating the involvement of the androgen receptor in imprinting is that a greater fraction of female mice heterozygous at the Tfm locus are resistant to the effects of neonatal androgen treatment than are wild-type females (Ohno, unpublished results; see Attardi et. al., 1976).

Goldstein and Wilson (1972) also showed

that prenatal injections of DHT to pregnant Tfm (o^+)/X ♀ carriers did not produce any in utero virilization of the Tfm (o^+)/Y♂ offspring. This observation by Goldstein and Wilson (1972) is consistent with a second theory of neonatal masculinization. According to this theory, estradiol is the agent responsible for masculinization and the effects of testosterone are secondary to the aromatization of testosterone by the hypothalamus (Naftolin et. al., 1975) and the subsequent binding of estradiol to specific receptors in the hypothalamus. There is considerable indirect evidence available in support of this theory. Goraki (1966) demonstrated that a smaller dose of estradiol was as effective as testosterone in masculinizing neonatal rats. Estradiol-17β is capable of restoring male behaviour in castrated red deer stag (Flecher and Short, 1974). Aromatizable androgens are most effective in inhibiting the development of cyclicity in female rats (McDonald and Doughty, 1974). In the castrated rat, DHT, a non-aromatizable androgen has a weak stimulatory action on sexual behaviour (Beyer et. al., 1973) while its combined administration with estradiol results in intense sexual activity in a number of species (Larsson et. al., 1973a, b; Baum and

Vreeburg, 1973; Feder et. al., 1974; Beyer et. al., 1975).

Estrogen binding proteins have been detected in the mouse hypothalamus (Fox, 1975; Attardi et. al., 1976) and furthermore, the number of estradiol binding sites are similar in brain hypothalamus (Fox, 1975) and cytosol (Attardi, 1976) from the normal male, female and Tfm/Y mice. Estradiol has also been reported not to induce neonatal masculinization in Tfm/Y mice (Ohno, unpublished results; see Attardi et. al., 1976). The presence of normal levels of the presumed estradiol receptor in Tfm/Y mouse hypothalamus extracts having low testosterone binding has been interpreted by Fox (1975) to suggest that conversion of androgen to estrogen is not essential for androgen action in the brain. This is in disagreement with the conclusion of Naftolin et. al. (1975) that "central androgen actions are in great part due to and require the aromatization of androgen to their centrally active principles - estrogens".

These theories to describe the mechanism of sex steroid action in the hypothalamus and other areas of the brain may each be correct to a certain extent.

Taken individually however, they may represent an over-simplification of the situation. Thus it is possible that there is a complex inter-relationship between the levels of androgen binding and estrogen binding, as well as other sex steroids such as progesterone. In considering brain receptor mechanisms for "sex steroids", androgens and estrogens are generally thought of as "male" and "female" hormones, respectively. This generalization may be misleading. Since cooperation and antagonism among androgens and estrogens occurs commonly in many tissues and species (Burrows, 1949) it is possible that androgen and estrogen effects on male and female brains result from modification of the predominant "male" and "female" ratios of androgens and estrogens (Fox, 1975). Furthermore since steroid hormones are known to affect synthesis of specific proteins via stimulation of the genome by the steroid-receptor complex (O'Malley et. al., 1969) it is also possible that androgen or estrogen receptor complexes may affect the synthesis of various enzymes such as those involved in the conversion of testosterone to estradiol, androstenedione to estrone, androstenedione to testosterone and estrone to estradiol.

In consideration of these inter-relationships

in the normal male, female and Tfm mice it has been shown that T and DHT binding to Tfm/Y♀ hypothalamus is reduced to 10-30% of normal male and female binding, while E_2-17β binding is essentially the same in male, female and Tfm/Y (Fox, 1975; Attardi et. al., 1976). Similar results have been found in normal male, female and Tfm/Y♀ kidney (Bullock and Bardin, 1975; Bullock and Bardin, 1974). From these studies and others it therefore seems reasonable to suggest that the primary genetic defect specified by Tfm is represented by a defective androgen receptor.

However, other alterations such as changes in steroid metabolism may also occur, secondary to the defect in the androgen receptor. In fact, it may be argued that the defective androgen receptor occurs secondary to some defect in steroid metabolism, although this would be unlikely. As shown in Fig. 22, Fig. 23 and Tables 21 and 22, total aromatization (E_1+ E_2-17β) of T by hypothalamus minces was 1.5 X greater in normal males than females and about the same as in Tfm (o^+) and Tfm (o^{hy}) hypothalamus incubations. Total aromatization by cortex minces was about 30%-50% of the hypothalamus levels. The major metabolite of T in normal male and

female incubations was E_1, while in both the Tfm (o^+) and Tfm (o^{hv}) incubations it was E_2- 17β. A preliminary report of these results has been presented elsewhere (Daley and Rosenfeld, 1976).

There has been only one other report of hypothalamic aromatization of androgens in the mouse (Naftolin et. al., 1975). In this paper, the conversion of A to E_1 was reported to be 2.9 X greater in male hypothalamus than female hypothalamus incubations, however because of the way in which these figures are reported it is not possible to directly compare the actual % conversions from starting material. It has also been suggested (F.Naftolin, see participant discussion Naftolin et. al., 1975) that aromatization in the Tfm/Y mouse is the same as normal males.

There are a number of possible explanations for the differences in the production of E_1 and E_2- 17β by normal males, female, Tfm (o^+) and Tfm (o^{hv}) hypothalamus. In normal males and females, the production of E_1 could be favoured by conversion of T to A and subsequent conversion to E_1 or alternatively by direct conversion of T to E_2 followed by 17β-oxidoreduction to E_1. Similarly, in Tfm (o^+) and Tfm (o^{hv}) incubations

production of E_2 most likely occurred *via* direct conversion from T, although the indirect route from T to A to E_1 to E_2 is also possible. An obvious extension of this work would be to compare the formation of E_2 from E_1 and vice versa in normal male, female and *Tfm* incubations.

APPENDIX

Chemicals

Diethyl ether reagent, A.C.S. was from Analar. Triton-X100 reagent was from Amersham. 2,2,2-tribromoethanol was from Aldrich Chemical. Hexane reagent, A.C.S. and methylene chloride reagent, A.C.S. were from Baker. Iso-octane reagent, A.C.S. and powdered silver oxide were from BDH. Glucose-6-phosphate dehydrogenase was from Boehringer Mannheim.

Gas chromatography packings 3% OV-17 on Chromosorb W(HP) 80/100 mesh and 1.5% SE-52 on Chromosorb 750 80/100 mesh were from Chromatographic Specialties. Cyclohexane reagent, A.C.S. and tetrahexylammonium hydroxide were from Eastman Chemicals. Liquid X-ray developer and Rapid Fixer were from Kodak.

Chloroform reagent, A.C.S. was from Mallinckrodt. Phosphomolybdic acid and silica gel F-254 sheets 0.25 mm thick were from E.Merck. Toluene reagent A.C.S. was from Matheson, Coleman and Bell. Acetic anhydride, benzene, ethyl acetate, heptane, methanol, methyl iodide, pyridine and propylene glycol reagents were from Fisher. Sodium hydroxide pellets and 2,5-diphenyloxazole (PPO)

were from Fisher. Medium 199 with glutamine and Hank's Balanced Salt Solution were from Grand Island Biologicals.

1,4-bis-[-2-5(phenyloxazolyl)] benzene (POPOP), scintillation grade was from Packard. Silyl-8 reagent, N_1O-bis (trimethylsilyl)-trifluroacetamide and chlorotrimethylsilane were from Pierce. Amylene hydrate was from Ptalty and Bauer and Sephadex LH-20 was from Pharmacia.

All non-radioactive steroids were from Steraloids and Sigma. Adenine trinucleotide phosphate, glucose-6-phosphate and nicotinamide adenine dinucleotide phosphate were from Sigma. Florisil was from Supelco.

Radiochemicals

4-^{14}C- androstenedione (58.8 mCi/mM), 6,7-^{3}H-estrone (40 Ci/mM), 6,7-^{3}H-estradiol (48 Ci/mM), 6,7-^{3}H-estriol (42 Ci/mM), 7α-^{3}H-testosterone (25 Ci/mM), 1,2-^{3}H-testosterone (52.8 mCi/mM) and 4-^{14}C--progesterone (52.8 mCi/mM) were purchased from New England Nuclear. 7α-^{3}H-androstenedione (30 Ci/mM) was from Amersham-Searle.

Animals

Tfm+(o^{+})+/y♀, Tfm+(o^{hv})Blo/y♀, Tfm+(o^{hv})Blo/+++, sxr/+♂ and +Ta++/+++, sxr/+♂ mice were obtained from stock maintained by Dr.S.Ohno, Department of Biology, City of Hope

National Medical Centre, Duarte, California. BALB/c ♂ and BALB/c ♀ mice were from Health Research, Buffalo, New York. All mice were housed in standard plastic cages in a controlled environment maintained at 22°C with lights on between 7 a.m. and 7 p.m. and were allowed access to laboratory chow and water ad libitum.

Equipment

An MSE Multex Centrifuge and IEC Model CS Centrifuge were used for low speed centrifugation. Homogenization was performed with a Pyrex AA25 homogenizer. Small samples were transferred using Eppendorf micropipettes and Hamilton 10 µl and 100 µl syringes. Materials were weighed on the Mettler P1200 top loader, Mettler H54 semimicro and Mettler M-5 microgram balances. Radiometer Copenhagen pH-meter 26 and pH-meter 51 were used for pH measurements.

Optical density was measured using a Beckmann DB-GT U.V. Spectrophotometer. Whatman #1 paper was used for paper chromatography. Silica gel F-254 0.25 mm thickness on plastic sheets 20 x 20 cm were from E.Merck. Thin-layer and paper chromatograms were observed under UV light on a Chromato-Vue UV Scanner from Ultra-Violet Products. A Nuclear Chicago Actigraph III Radio-

chromatogram Scanner was used with 1.3% butane in helium and a collimator slit width of 6 mm for scanning paper chromatograms. Gas chromatography was done using a Varian Series 2100 GC and a Packard Model 823 GC. Mass spectroscopy was done using a Varian 2700 GC coupled to a Varian MAT CH7 Mass Spectrometer and with a Consolidated Electrodynamics Model 21-110B Mass Spectrometer. Radioactivity was measured in 5 dram glass vials from Wheaton Products using a Beckmann LS-233 Scintillation Counter.

Solvent extractions were performed using a Burrell Wrist Action Shaker Model 75. Incubations were done in a Dubnoff Metabolic Shaker.

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