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IN VITRO METABOLISM OF C_{19} STEROIDS IN HUMAN ENDOMETRIUM

by

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ABSTRACT

In order to quantitate the extent of intracellular or metabolic conversions of C_{19} steroids in human endometrium, specimens of proliferative and secretory tissue were superfused at a constant rate with several pairs of labeled compounds at low concentrations.

From these superfusions it was determined that 10 to 20% of the dehydroepiandrosterone sulfate which interacts with the endometrium is hydrolyzed to dehydroepiandrosterone, regardless of the phase of the menstrual cycle.

Testosterone and androstenedione are interconverted in endometrial tissue by 17β hydroxysteroid dehydrogenase with a preference toward oxidation. Both the oxidative and reductive activities of this endometrial enzyme increase during the luteal phase of the menstrual cycle, likely under the influence of progesterone. The testosterone taken up by endometrial cells is mostly oxidized to androstenedione and released from the tissue as androstenedione, while the androstenedione taken up by the tissue is mostly removed from the tissue by diffusion.

The physiologically active androgen dihydrotestosterone is formed in endometrial tissue. It arises from the testosterone entering the cells which is directly 5α reduced (about 1%) and from the androstenedione which is first 5α reduced to androstanedione and then 17β reduced to dihydrotestosterone. The intracellular concentrations of these two precursors, testosterone and androstenedione, determine the relative importance of the two pathways of 5α reduction. With a tissue concentration of testosterone larger than the tissue concentration of

androstenedione, most of the testosterone is directly 5α reduced to dihydrotestosterone. Under these conditions, most of the androstenedione is still metabolized to dihydrotestosterone through androstanedione.

Other C_{19} metabolites produced by endometrial tissue are androsterone and 5α-androstane-3α,17β-diol.

Human endometrial cancer cells in culture contain the same enzymes capable of metabolizing C_{19} steroids as are found in normal human endometrium. The HEC-1 cell line and a unique cell culture derived from an epithelial carcinoma of the uterus of a phenotypic female with an XY genotype metabolized testosterone to androstenedione, androsterone and 5α androstane-3α,17β-diol. The HEC-1 cells preferentially produced 5α-androstane-3α,17β-diol and the XY cell culture preferentially produced androstenedione.

The results of these experiments with normal human endometrial tissue demonstrated that the conversion of the main circulating C_{19} steroids in women, i.e. dehydroepiandrosterone sulfate and androstenedione to dihydrotestosterone, the compound considered to be the true intracellular androgen, is very small.

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Abbreviations Used in This Text

G.B.SS.....Gey's balanced salt solution from Grand Island Biological Co.
R.I.....differential refractometry
17β HSD.....17β hydroxysteroid oxidoreductase
3α HSD.....3α hydroxysteroid oxidoreductase
Δ^5-3β-ol HSD.....Δ^5-3β-ol hydroxysteroid oxidoreductase

DS.....dehydroepiandrosterone sulfate
D.....dehydroepiandrosterone
T.....testosterone
Δ.....androstenedione
Adione.....androstanedione
DHT.....dihydrotestosterone
E_1.....estrone
E_2.....estradiol
3α,17β-Adiol.....5α-androstane-3α,17β-diol
3β,17β-Adiol.....5α-androstane-3β,17β-diol

α_A.....the fraction of the perfused tracer A which enters the tissue
β_A.....the fraction of the perfused tracer A which is released back to the perfusate
γ_{AB}.....the fraction of the perfused tracer A which is released by the tissue as compound B
γ_{AM}.....the fraction of the perfused tracer A which is released by the tissue as metabolite M
ρ_{AB}.....the fraction of the perfused tracer A which is converted in the tissue to compound B
$(\beta/\alpha)_A$.....the fraction of the perfused tracer A entering the tissue that is released unmetabolized to the medium
γ_{AB}/α_A.....the fraction of the perfused tracer A which enters the tissue that is released to the medium as compound B
$(T/M)_A$.....the ratio of the intracellular concentration of tracer A (cpm/g) to the concentration of tracer A in the medium (cpm/ml)
$(^3H/^{14}C)_{At}$.....the isotope ratio in intracellular compound A
$(^3H/^{14}C)_{supf}$.....the isotope ratio in the inflowing medium

v_{OA}.....the rate of entry of superfused A into cells
v_{AO}.....the rate of irreversible removal of intracellular A
v_{AB}.....the rate of intracellular conversion of A to B
$v_{A_{\bar{p}}}$.....the rate of release of A from the tissue

Abbreviations - continued

v_{AM}.....the rate of release of A from the tissue as a metabolite
PR_A.....the rate of "de novo" formation of intracellular A
$a_{At}^{^3H}$.....the intracellular specific activity ^3H-A (cpm/pmol)
$a_A^{^3H}$.....the specific activity of ^3H-A in the inflowing medium (cpm/pmol)
$c_{At}^{^3H}$.....the intracellular concentration of ^3H-A (cpm/g)
$c_A^{^3H}$.....the concentration of ^3H-A in the inflowing medium (cpm/ml)
$c_{A_p}^{^3H}$.....the concentration of ^3H-A in the superfusate (cpm/ml)
c_{At}.....the intracellular concentration of compound A (pmol/g)
c_A.....the concentration of compound A in the in flowing medium (pmol/ml)
ϕ.....flow rate of medium (ml/hr)

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I. INTRODUCTION

1. HISTORICAL BACKGROUND

Since the isolation of androsterone from male urine by Butenandt (1) and its later synthesis from cholesterol by Ruzicka and co-workers in 1934 (2) scientists have been studying the effects of androgens upon the uterus. The first to publish was Vladimir Korenchevsky at the Lister Institute in London. In 1935 and 1936 (3,4), he presented the results of experiments on rats ovariectomized before sexual maturity which had been injected for 26 days with estrone, synthetic androsterone and synthetic androstanediol in doses up to 316 mg per day for the androgens and 6 μg three times weekly for estrone. By weighing and histologically examining the uteri, he concluded that large doses of androsterone had a very slight effect upon the restoration to normal weight and appearance of the uteri, large doses of androstanediol were much more effective but estrone was the most effective. He found that estrone and large doses of either androgen produced the nearest approach to normal of the uterus in weight and appearance.

Testosterone was first isolated from bull testis and crystallized by E. Laquer et al (5) in 1935. At practically the same time, Butenandt and Hanisch (6) and Ruzicka and Wettstein (7) announced the artificial preparation of testosterone. Using Ruzicka's artificial testosterone, Korenchevsky et al repeated their experiments with ovariectomized rats (8,9). With doses of testosterone of 700 μg per day for 21 days, they reached similar conclusions to those above, finding that testosterone restored the uterus to a more normal weight than androsterone, the change being somewhat less than that obtained

with androstanediol. Histological examination of the uteri revealed that the mucosa was less developed in relation to the myometrium than in the normal diestrus uterus, the two layers being about the same thickness. When estrone was injected with the testosterone, the effect was more pronounced. Testosterone propionate had been synthesized around this time so Korenchevsky compared it with testosterone (10,11,12). Using the same protocols as in his early experiments, he found that testosterone propionate at doses of 700 µg produced changes similar to testosterone but with greater hypertrophy. Comparing testosterone propionate with the dipropionate of the enol form of testosterone, and estradiol, estradiol 3 benzoate-17N butyrate and estradiol dipropionate (13,14), he found that testosterone dipropionate and estradiol benzoate-butyrate were the most stimulatory. The value of using esterfied hormones became apparent.

Korenchensky and his group were not the only investigators in this field at the time but they were the most prolific. Others who studied the effects of large doses of testosterone upon the rat uterus were R. Deanesley and A.S. Parkes (15), Warren O. Nelson and Charles G. Merckel (16), Ludwig G. Browman (17), J.B. Broaksby (18) , U.J. Salmon (19) and Mazer and Mazer (20). They uniformly found that doses of 500 µg or larger of testosterone or testosterone propionate given to intact or ovariectomized mature or immature rats for a month or less caused hypertrophied uteri.

Animals other than rats were studied also. With immature and ovariectomized adult rabbits, similar conclusions were made. S.L. Leonard et al (21) found that in young castrated rabbits after 6

days of estrone priming, 7.5 to 20 mg of testosterone propionate for 5 days prevented castration atrophy of the uteri. Using a similar method , J.M. Robson (22) found "proliferation almost of the order of that seen at the height of pseudo-pregnancy." In the immature intact rabbit, 20 mg of testosterone over 5 days produced a response similar to 2 μg of estrone (23).

In intact adult and adolescent monkeys (24,25,26), daily multi-milligram doses of testosterone or testosterone propionate delayed bleeding beyond the expected end of the menstrual cycle. Engel and Smith (27) concluded that "the interval between cessation of testosterone propionate treatment in estrogen pretreated monkeys and the appearance of bleeding is similar to that of estrogen withdrawal. The endometrium of such estrogen-testosterone treated monkeys is essentially a proliferative type....."

The problem of prolonging the activity of the steroids was approached in several ways. First, the compounds were dissolved in oil with the amount of oil increased to delay the rate of absorption. Then, the hormones were esterfied with aliphatic esters to decrease metabolic deactivation. The ultimate solution for Deanesly and Parkes (28) was the subcutaneous implantation of a compressed tablet of pure dry hormone which could be recovered, weighed and reused. Using this technique on adult guinea pigs, they implanted 20 mg of testosterone propionate for 142 days. The average amount absorbed was 16 mg, about 17% per month (29). At autopsy, the uterus was hypertrophic. Similar results were achieved with ovariectomized rats.

Since Korenchensky's first experiments with androsterone and

androstanediol, androgens other than testosterone have been studied for their effect upon the uterus. Δ^5 androstenediol and Δ^4 androstenedione were regarded at this time as possible intermediate links in the formation of testosterone from cholesterol and therefore worthy of study. Using doses of from 200 to 3000 µg for each compound per day for 21 days in castrated immature rats, Korenchensky concluded (10) that both compounds equally caused slight hypertrophy of all layers of the uterus. Other groups agreed doing similar studies with Δ^4 androstenedione (16,30) but not with Δ^5 androstenediol which they found to be more uterotropic (23,31). Dihydrotestosterone, methylated or ethylated at C-17, also was injected in mg doses for several days to immature or ovariectomized adult rabbits and was found to cause as much proliferation as equal doses of testosterone (32).

The routine experimental procedure during this period, the 1930's, had been to inject the animals for less than a month. Korenchensky again was in the avant garde in realizing that experiments of longer duration were needed. He therefore repeated his earlier experiments on intact and ovariectomized immature rats, injecting them for three months with androgens and estradiol dipropionate or benzoate butyrate (33,34). Androgens were given in 5 doses per week totalling either 2.25 or 7.5 mg and estradiol was given in 3 doses per week totalling 90 µg. With testosterone propionate in ovariectomized rats he found no essential difference as compared to the earlier experiments , except that the uteri were larger. However, when testosterone propionate and estradiol were injected together, there was squamous metaplasia and a very pronounced cystic hyper-

plasia of the uterine glands. In intact rats, his results were unexpected. With the 7.5 mg dose, a "gigantic" uterus was obtained but with the 2.25 mg dose, the uterus was decreased in weight and size as compared with the normal controls.

This was not the earliest report of an inhibiting action of testosterone on the female genital tract. In 1936, J.M. Robson (35) reported that 400 μg of testosterone inhibited the expected estrus reaction of 0.1 μg of estrone in the vaginas of mature ovariectomized mice. This effect of small doses of testosterone propionate on rats was noted also by others and was explained as due either to the lack of ovaries with corporea lutea or to pituitary inhibition of the ovary from prolonged androgen. In one study (36), normal cycling rats given 200 μg of testosterone propionate daily for 10 days had swollen uteri with tall columnar epithelium, while ovariectomized mature rats given the same treatment had smaller uteri with low epithelium. In another study (37), immature rats (weighing 30-40 g) receiving 500 μg of testosterone propionate three times a week for 14 1/2 weeks and adult rats receiving the same dose for 65 days, (all) had uteri which weighed 25% less than the controls. The conclusion here was pituitary inhibition. In an attempt to settle the question of the mode of action of testosterone on the uterus, Ira T. Nathanson et al (38) studied the effect of a single dose of testosterone propionate varying from 2.5 to 10 mg upon the uteri of intact rats, ovariectomized immature rats, hypophysectomized immature rats and hypophysectomized ovariectomized immature rats. They found that the uteri of all of these animals were enlarged with increased stroma,

vascularity and glands. The only difference was one of degree in that the intact rats had more uterine glands. The conclusion therefore was that testosterone directly stimulates uterine growth.

Still results continued to be published which showed that testosterone had both a stimulating and inhibiting effect upon uterine growth. Joseph Velardo studied (39,40,41) the effect of various doses of testosterone with and without estradiol on mature ovariectomized rat uteri and concluded that in daily doses of 2 to 5 mg testosterone affected uterine growth in a way indistinguishable from that produced by three daily injections of 0.1 μg of estradiol, however, the same dose of estradiol and 0.5 to 1.5 mg of testosterone given simultaneously caused a significant reduction in uterine growth. Others (42,43) agreed that low doses of testosterone depressed estrogen induced growth while larger doses had no effect (44) or caused uterine hypertrophy (45).

In all of these preceding studies, the uteri were weighed and usually examined histologically. It was Leonard J. Lerner who brought a new dimension to the study of the effects of androgens on the uterus by adding other criteria in order to elucidate the mechanism of action of uterotropic steroids. He selected specific biochemical end points which reflect growth and functional state, one, protein synthesis as reflected by measuring nitrogen and nucleic acids and two, energy metabolism, as reflected by measuring three enzyme systems, glucose-6-phosphate dehydrogenase, malic dehydrogenase and isocitric dehydrogenase (46). He found that testosterone propionate in doses of 0.5 to 12.5 mg increased in a dose-dependent

fashion the total uterine RNA, DNA and the RNA to DNA ratio. It also increased the activities of the three enzyme systems. He concluded that the effects of these doses of testosterone propionate were similar to smaller doses of estrogen on the biochemical end points but that there were morphological differences. In an attempt to elucidate the differences between the actions of estrogen and testosterone upon the uterus, Lerner (47) studied the effects of hormone antagonists on the changes induced by the steroids. These antagonists were Mer 25 (1-[p-2-diethyl-aminoethoxyphenyl]-1-phenyl-2-p-methoxyphenyl ethanol) (48), which opposes the activity of estrogens and A-norprogesterone (49,50), an androgen antagonist. Mer 25 at high doses completely reversed the estrogen-induced change in uterine weight, morphology and biochemical parameters. It partially inhibited the testosterone propionate-induced increase in uterine weight but did not affect the biochemical or histological changes. A-norprogesterone reduced the testosterone propionate-induced uterotropic response but did not affect any of the induced enzyme activities. It did affect the histological picture causing a single layer of low columnar epithelium lining the lumen instead of the high columnar epithelium usually seen after testosterone. However, it did not appear to antagonize the hypertrophy of the stroma or myometrium. Other antagonists have been used in the attempt to separate estrogenic from androgenic causes of uterine growth. Estr-4-ene-3-one-spiro-17β-2'-tetrahydrofuran, an antiestrogen that partially inhibits the uterine weight increases caused by estrogen in the mouse, but has no affect upon the testosterone-induced weight increase (51).

Clomiphene (2-[p-(2-chloro-1,2-diphenylvinyl) phenoxy] triethyamine) also failed to prevent the changes induced by testosterone in female rats (52). Several antiandrogens, DIMP (N-[3,5-dimethyl-4-isoxazolyl-methyl] phthalimide) (53), BOMT (6α-bromo-17β-hydroxy-17α-methyl-4-oxa-5α-androstan-3-one) (54) and SCH 13521 (4'nitro-3'-trifluoro-methyl-isobutyranilide) (55) have been shown to prevent the uterotropic response to testosterone in rats.

Contemporary with Lerner's experimentation, scientists for the first time showed that in the uterus estrogens are bound to cytoplasmic receptor molecules and then transported into the cell nucleus (56). Since evidence has been published that testosterone appeared not to compete with estradiol for binding sites (57), George Giannopoulos began to look for the mechanism of action of testosterone in the uterus and isolated a specific binding protein for it in the immature rat uterine cytosol which translocates to the nucleus (58, 59). He found 5α dihydrotestosterone a weak competitor for this binding site. At the same time, Jungblut et al (60) isolated a 5α dihydrotestosterone receptor in immature calf uterine cytosol. They did not look for testosterone binding. The question of the specificity of androgen binding in the uterus was possibly answered by Heyns, Verhoeven and De Moor (61,62,63,64) who concluded from their experiments that 5α-dihydrotestosterone was only apparently weaker in binding than testosterone because of indirect factors. These factors were the extensive metabolism of dihydrotestosterone to 3α-androstanediol which only weakly binds to the cytosol receptor, the considerably higher nonspecific binding of dihydrotestosterone as compared to

testosterone and finally experimental procedures involving the method by which the hormone-bound receptor is separated from the rest of the cytosol proteins. Since the dissociation of the dihydrotestosterone receptor complex is considerably slower than that of the testosterone receptor complex, it is favored by 18 hours of sucrose density gradient centrifugation as compared to the shorter time it takes to perform gel filtration or ammonium sulfate precipitation.

Receptors for either testosterone or 5α-dihydrotestosterone have been reported also in human endometrium by several groups of investigators (65,66,67, 68) and in human myometrium (69).

Since cytosol and nuclear receptors for estrogen in the target cell have similar properties (70) and since estradiol in vivo induces a simultaneous decrease in cytoplasmic receptor and increase in nuclear receptor (71), the assumption was made that the nuclear receptor was either part of or the complete cytosol receptor which had been translocated (56,71). With this in mind, Rochefort et al decided to study the specificity of various compounds in transfering the rat uterine estrogen receptor to the nucleus by measuring their in vitro effect upon the number of specific estrogen binding sites in the cytosol and in the nucleus (72). The compounds were estrone, nafoxidine and several androgens. Both estrone and nafoxidine bound to the cytosol receptor and moved into the nucleus as was expected but to their surprise, the androgens, especially dihydrotestosterone and testosterone in 1 μM amounts, also moved the estradiol receptor into the nucleus with an accompanying depletion in cytosol receptor. They termed this "transfer activity" and suggested that the androgens

induced the nuclear receptor formation through modification of the nuclear membrane.

As it had already been determined that the rat uterus has an androgen receptor, Thomas Ruh (73) wanted to determine if this receptor was necessary for the androgen-induced nuclear accumulation of the estrogen receptor. He concluded that it was not because the antiandrogens BOMT and DIMP did not inhibit the dihydrotestosterone-induced nuclear uptake, however, antiestrogens C.I. 628 and U-11, 100 A did. Next, he questioned whether the estrogen-induced specific uterine protein (I.P.) which had been reported from in vitro experiments in rats (74) could be stimulated in vitro by testosterone and dihydrotestosterone. He found that it was induced and that its induction was not inhibited by BOMT but was inhibited by C.I. 628 and U-11, 100 A. This I.P. was indistinguishable from that stimulated by estradiol but, for the amount produced, it required 200 times more dihydrotestosterone and 3000 times more testosterone than estradiol (75).

If the androgen receptor is not responsible for the androgen-stimulated nuclear accumulation of the estrogen receptor and the production of I.P., is it responsible for the uterotrophic effect of androgens? Marcel Garcia and Henri Rochefort (76) suggest from their experiments that it is not. They found that 100 µg of dihydrotestosterone induced maximal occupation of the androgen receptor sites but was ineffective in translocating the estrogen receptor or in increasing general protein synthesis whereas doses of dihydrotestosterone larger then 3 mg translocated the estrogen receptor and induced the

I.P. From this, it appeared that the uterine response depended more upon the nature of the receptor translocated than the nature of the hormone-bound to the receptor. Their next question was how does the androgen translocate the estrogen receptor (77). Studying the incubation of testosterone and ^{3}H-estradiol for various periods of time with rat or calf uterine cytosol, they found that testosterone was too weak to prevent estrogen binding at equilibrium but was strong enough to decrease the association rate of ^{3}H-estradiol to its receptor. To investigate the in vivo mechanism of estradiol receptor translocation, they injected a 0.4 or 15 mg dose of dihydrotestosterone in oil and then using an exchange technique, labeled the cytosol and nuclear extracts with ^{3}H-estradiol. By doing this, they were able to demonstrate that 3 to 5 hours after the dihydrotestosterone injection, the cytosol estrogen receptor content decreased and the KCl nuclear extract content increased. As compared to an estrogen-induced translocation, this translocation was delayed and transient. From these experiments, it was concluded that the mechanism involved weak affinity binding of androgens to the estradiol receptor which appeared sufficient to induce the "activation" process leading to nuclear translocation. Their conclusions are supported further by evidence of an affinity of 5α-dihydrotestosterone for the rat pituitary cytosol estradiol receptor (78) and for the human myometrial cytosol estrogen receptor (79).

At the same time, Schmidt, Sadler and Katzenellenbogen also were studying the actions of androgens on the uterus and concluded from their experiments that the in vivo and in vitro mechanisms were

different. When in vivo and in vitro tissue uptake of androgens is equivalent, in vivo androgens were unable to translocate the estrogen receptor system as occurs in vitro (80). Also, the medium in which the androgen is injected was important. When 5 or 10 mg doses of dihydrotestosterone were injected in oil, nuclear translocation and cytoplasmic depletion of the estrogen receptor occurred with a concomitant uterotropic response. which was resistant to antiandrogen antagonism. However, with the same dose of androgen in water soluble dimethylsulfoxide, there was little estrogen receptor movement and the uterine growth was almost completely eliminated with an antiandrogen (81).

This effect of high doses of androgen on the estrogen receptor is not limited only to rats but has been reported also in the human breast cancer cell line MCF-7 (82) where progesterone receptor synthesis is stimulated.

Thus in unphysiologic doses, testosterone and 5α-dihydrotestosterone act through the estrogen receptor, but what is the evidence for actions through the androgen receptor? Histological differences between an estrogen and androgen-stimulated uterus are the differences most often emphasized. Korenchensky first described in testosterone-treated ovariectomized rats, a myometrium more developed than the mucosa due to hypertrophy of the loose connective tissue and blood vessels between the circular and longitudinal muscle layers (9). There was an increase in the number but especially in the size of the muscle cells (10). This observation of a greater stimulation of the muscle layers by androgens as compared to estrogens has been

supported by many other investigators (46,47,55,83,84). As Beyer and Komisaruk (85) said, "it is tempting to speculate that the uterine weight increase is mainly due to the anabolic effect of these androgens on the myometrium."

The only other reported differences between estrogen and testosterone stimulated uterine growth relate to RNA synthesis and have been described in a series of experiments by N.A. Yudaev and B.V. Pokrovskii (86,87,88,89). Studying the incorporation of radioactive uridine or adenine into RNA in ovariectomized rat uteri equally hypertrophied by estradiol or testosterone phenylpropionate, they found a quantitative increase in incorporation of the isotope in the testosterone stimulated uteri, as compared to the estradiol stimulated uteri. In other experiments, after pulse labeling with ^{3}H-uridine for estradiol stimulated rat uteri and ^{14}C uridine for testosterone stimulated uteri, they concluded that the radioactivity of the fast-labeling nuclear and cytoplasmic RNA sedimenting from 6-18S was considerably higher in rats given estradiol as compared to those given testosterone. Their final experiments involved saturation and competitive hybridization of DNA with ^{3}H-RNA from androgenized and estrogenized uteri. In saturation hybridization, the degree of hybridization of nuclear ^{3}H-RNA from the androgenized uteri exceeded the level of hybridization of the analogous ^{3}H-RNA from the estrogenized uteri. With competitive hybridization, they found a difference in the degree of competition of the RNA from estrogen stimulated tissue on one hand and androgen stimulated tissue on the other. The results from these three groups of experiments confirmed for them

their hypothesis that testosterone has an independent role in regulating biosynthetic processes in the uterus.

If the action of multimilligram doses of androgen is mediated through the estrogen receptor, the mechanism for inhibition of uterine growth by smaller doses of testosterone is not known. Early theories that the effect was mediated through the pituitary have been disproved (21,34,36,37,38). It has been suggested that (90) testosterone might act like a temporary agonist which delays the cytosol estrogen receptor replenishment. However, why this complex would be less efficient in stimulating growth than estradiol binding to its receptor is difficult to explain. Maybe, this lack of efficiency is due to different binding characteristics such as a high rate of dissociation because of the inability to transform the estrogen receptor into a slowly dissociating complex or maybe there is a discrete difference in the conformational change in the receptor when it is bound to testosterone and then to the chromatin acceptor sites.

Androgens may also be antiestrogenic inhibiting uterine growth by binding to the progesterone receptor (91,92,93). Thus, inhibition of uterine growth caused by testosterone or dihydrotestosterone could be mediated through one of three receptors, the estrogen, progesterone or androgen receptor.

An important remaining question is what is the effect of physiological doses of androgen upon the uterus as mediated through the androgen receptor? There are many fewer androgen receptors in the rat uterus than there are estrogen receptors. The most recently reported concentration is in the range of 125-175 fmol/mg of cytosol

protein (81). This is a somewhat higher value than first reported (58,64,91). In the human endometrium, the level is estimated at between 200 and 500 fmol/mg of protein (65,66). The dissociation constants for all of these receptors are in the tenth of a nM range.

The in vivo dose of testosterone or dihydrotestosterone which interacts only with the rat uterine androgen receptor is reported to be between 100 and 300 μg. At the lowest, 100 μg, Garcia and Rochefort (76) found no increased incorporation of ^{3}H-leucine into proteins, at 300 μg, Schmidt and Katzenellenbogen (81) found a uterine weight increase of about 25% more than controls that was abolished by antiandrogens but not by antiestrogens.

In summary, both estrogens and androgens cause an increase in uterine weight in rats which can be abolished by antiestrogens and antiandrogens, respectively; thus the assumption is made that the mechanism of action of the estrogens and androgens is different. This is further supported by studies which have shown a high affinity, low capacity protein binder, i.e., a receptor for dihydrotestosterone and testosterone in both rat and human uteri. The principally observed differences between the estrogenic effects of androgens and estrogens upon rat uterine growth have involved the myometrium. Vladimir Korenshevsky (9,10) said of the androgen stimulated uterus "..... the myometrium was thicker than the mucosa, due chiefly to a hypertrophied layer of loose connective tissue and blood vessels between the circular and longitudinal layers of the uterus..... The hypertrophy of the tissues is explained by the increase in both number and size of the cells, the latter chiefly in the myometrium."

Nelson and Gallagher (83) described these uteri as showing a marked increase in connective tissue and smooth muscle and Groome (84) said that the uteri were highly vascular with thickening of muscle and mucosa. Neri et al (55) stated "histological examination of uterine sections indicated that these androgens (testosterone, androstenedione, and dihydrotestosterone) increase the size of the lamina propia primarily and the myometrium to some extent." Lerner (46) first states that the histological responses evoked by the two compounds (estradiol benzoate and testosterone propionate) are distinctly different but in a later publication (47) he says "... the histological picture (of testosterone propionate) resembles that seen with estrogen treatment .... although the mucosa looks pseudostratified." In comparison, estrogen is described as stimulating mostly growth of the epithelial glands of the endometrium.

Antiestrogenic effects of androgens in rats, where dihydrotestosterone can suppress the growth normally caused by estrogen also have been reported. The exact mechanism of this effect is not understood but estrogen receptor binding studies point to the possibility that testosterone and dihydrotestosterone in large doses bind to the estrogen receptor and behave like short acting antiestrogens such as estriol or dimethylstilbestrol. However, a recent report (94) concluded that at 10^{-8} M testosterone or dihydrotestosterone inhibited the effect of estrogens on the induction of the progesterone receptor in the MCF-7 human breast cancer cells. This effect of the androgens was antagonized by cyproterone. Thus, at this time, there are more questions concerning the physiological function of androgens in the

uterus than there are answers. This makes the function of androgens in endometrial tissue an interesting and challenging problem.

2. Purpose of the Study

One question which is important to answer, when one is considering the possibility that androgens have a physiological function in endometrial tissue, is whether the main circulating C_{19} steroids in women (e.g. dehydroepiandrosterone sulfate, dehydroepiandrosterone and androstenedione) are significantly converted to testosterone and dihydrotestosterone, compounds which are considered to be the effectors of androgen action in target tissue.

Dihydrotestosterone circulates in human plasma at a concentration of approximately 0.2 ng/ml (95,96,97). It is strongly bound to the testosterone-estradiol-binding globulin (TeBG), the dissociation constant estimated by steady state polyacrylamide gel electrophoresis at 37 C is 2.5×10^{-10} M (98), as well as being bound to serum albumin. Testosterone at a plasma concentration of approximately 0.4 ng/ml (95,96,99) also is strongly bound to TeBG. However, there are other C_{19} steroids in the plasma which are more available to the endometrium because of their higher plasma concentrations and their weaker binding to plasma proteins, such as dehydroepiandrosterone at 4.5 ng/ml (95,99,100,101,102), dehydroepiandrosterone sulfate at 2000 ng/ml (95,101,103,104) and androstenedione at 1 to 2 ng/ml of plasma (95,99,104,105). Table 1 lists the plasma concentrations of C_{19} steroids in pre and postmenopausal women. In addition to these comparatively high plasma levels, there is evidence that endometrial tissue concentrates steroids, including dehydroepiandrosterone and testosterone, to a significantly higher level than that in plasma. Assuming that 1 ml of plasma is equivalent to 1

Table 1

Plasma C_{19} Steroid Concentration (ng/ml)

Steroid	Premenopausal Mean	Premenopausal S.D. (range)	Postmenopausal S.D.	Reference
Dehydroepiandrosterone Sulfate	2238	560	442 63	95
	2020	108	764 28	103
	2000	370		104
	1910	650		101
Dehydroepiandrosterone	4.6	1.9	2.4 1.2	102
	4.6	2.2		101
	4.56	1.00	1.46 0.14	95
	4.2	(0.9-10.9)		100
	4.1	(2.2-6.3)		99
Androstenedione	2.2	(0.8-4.8)		99
	1.8	0.095	0.92 0.38	175
	1.25	0.11	0.27 0.02	95
	1.1	0.15		104
			0.83 0.13	105
			0.47	175
Testosterone	0.59	(0.2-1.7)		99
	0.52	0.12		221
	0.43	0.12	0.41 0.06	96
	0.40	0.16		213
	0.30	(0.23-0.33)		106
	0.30	0.3		104
	0.27	0.6	0.19 0.03	95
	0.27	0.015	0.215 0.01	175
5α-Dihydrotestosterone	0.18	0.07		96
	0.17	0.04	0.09 0.01	95
	0.14	0.5		97
Androsterone	0.3	(0-0.63)		100
Androstene-3β,17β-diol	1.17	0.16		216
	0.90	0.2		215
	0.75	0.1	0.33 0.05	95
	0.68	0.24		222
	0.55	0.22	0.40 0.23	214
5α-Androstene-3β,17β-diol	0.52	0.18		218
	0.3			217
5α-Androstene-3α,17β-diol	0.26	0.005		219
	0.17	0.098		220
	0.11	0.033		218

gram of tissue, Guerrero et al (106) extracted twenty times more dehydroepiandrosterone and eight times more testosterone from endometrial tissue than from peripheral blood. Therefore, the level of a steroid available as substrate for enzymatic conversion to biologically active dihydrotestosterone may be higher in tissue than in plasma.

The purpose of this project has been to study the metabolism of naturally circulating androgens by proliferative and secretory human endometrial tissue in order to elucidate the normal metabolic pathways and to learn whether the endocrine states of the tissue vis-a-vis the menstrual cycle has any effect upon this metabolism. It is known that estrogen metabolism in the endometrium is influenced by the increased concentration of progesterone during the secretory half of the menstrual cycle (107,108). Progesterone stimulates the activity of 17β hydroxysteroid dehydrogenase so that there is greater oxidation of estradiol to estrone. There is evidence that testosterone, Δ^5 androstenediol and androstenedione are substrates for this same enzyme (108,109) so possibly the oxidation of testosterone to androstenedione, a biologically less active androgen is increased also during the secretory phase. Whether 5α reductase activity in the endometrium is affected by the phase of the menstrual cycle has not been established, as Rose et al (110) found no difference between proliferative and secretory endometrium, while Pollow et al (111) found the highest values for 5α reductase activity during the early proliferative phase. Therefore, given that the importance of regulation of enzymatic activities in controlling levels of

active steroids in target cells is established, knowledge of the extent of metabolic conversions of C_{19} steroids in the endometrium during the cycle will lead to a better understanding of their potential biologic activity.

3. Experimental Approach

With fresh endometrial tissue available from surgery, the most logical way to approach this study was the classical technique of tissue incubation with isotopically labeled steroids. This tissue could be used either as tissue slices or in broken cell preparation. I chose the former because a broken cell preparation used to study enzymatic activity would require the use of cofactors and the relative proportion of reduced or oxidized metabolites would vary greatly depending upon the nature of the cofactors added to the incubation medium. Pollow et al found that NAD^+ was the cofactor of choice for 17β dehydrogenase in the endometrium where it oxidized estradiol three times more rapidly than $NADP^+$, while in the same tissue 5α reductase preferred NADPH (111). Furthermore, a broken cell preparation would not be an approximation of normal physiology, since the structural unity and therefore the normal interaction between the cell's components is destroyed.

The choice of the type of incubation to use was decided by the obvious advantage of superfusion, i.e., continuous unrecycled perfusion of the tissue, over batch incubation. With batch incubation, one has a static view of metabolism because at any point of time during the incubation a sampling of the steroids in the tissue or the medium will reflect only the net influx and efflux of that compound from the tissue as a result of the concentration of the substrate or product in the tissue and the medium at that time. In order to determine rates of metabolism, a dynamic view, it is preferable to have the tissue exposed to a constant concentration of

substrate over a constant period of time which is impossible with batch incubation unless the concentration of substrate is so large that the specific activity of the precursor and the product are nearly constant throughout the incubation. With this large concentration of substrate there is always the possibility that once the enzyme is saturated, the excess substrate or large amount of the product may distort the normal pattern of metabolism. If one wishes to use physiological tracer amounts with a batch incubation, one would have to take frequent aliquots of the compounds in order to follow a changing rate of metabolism as the concentration of the substrate decreases. This is technically impractical and makes rate calculations difficult. Therefore, granted that tracer amounts of steroid are desirable and that one wants to maintain a constant near physiologic concentration of steroid in the tissue, the best method to achieve this steady state is by superfusion. With it there is an equal rate of diffusion of the steroid into and removal from the cells leading to a constant intracellular steroid concentration. Using this superfusion with two metabolically interconvertable isotopes, one is able to quantitate several parameters of metabolism. This has been shown by Gurpide and Welch, who also used endometrial tissue (112).

The value in using two metabolically interconvertible isotopes is that one can distinguish the fraction of a steroid A that enters the tissue and is released back to the medium from the fraction of the steroid which by-passes the tissue. This is done by labeling the steroid A with tritium and adding to the perfusion medium a ^{14}C

labeled precursor, steroid B. Both of these steroids will be taken up by the tissue and the intracellular conversion of steroid B to steroid A will produce in steroid A a $^{3}H/^{14}C$ ratio. The steroid A released from the tissue will have this ratio, whereas the steroid which by-passed the tissue will contain only one isotope. Therefore, one can distinguish and quantitate the fraction of the steroid which entered the tissue and was released from the fraction which by-passed it. With this information, it is possible to quantitate the rate of conversion of steroid B to steroid A. If steroid A is also convertible to steroid B, then knowing the $^{3}H/^{14}C$ ratio in steroid B, makes it possible to distinguish in a similar fashion the fraction of steroid B which is released from the tissue from the fraction of the steroid which by-passed the tissue. With this ability to distinguish between what enters the tissue and what by-passes it, one can calculate the rates of uptake by the tissue of these two intrachangeable steroids, their rates of intracellular conversion and their rates of release from the tissue. One can also calculate the fraction of these steroids which are metabolized to compounds other than each other. This is some of the information to be obtained from these experiments.

The theoretical aspects of these calculations have been published in "Methods in Enzymology" (113) and the equations related to these experiments are described in sections to follow:

4. Description of Studies

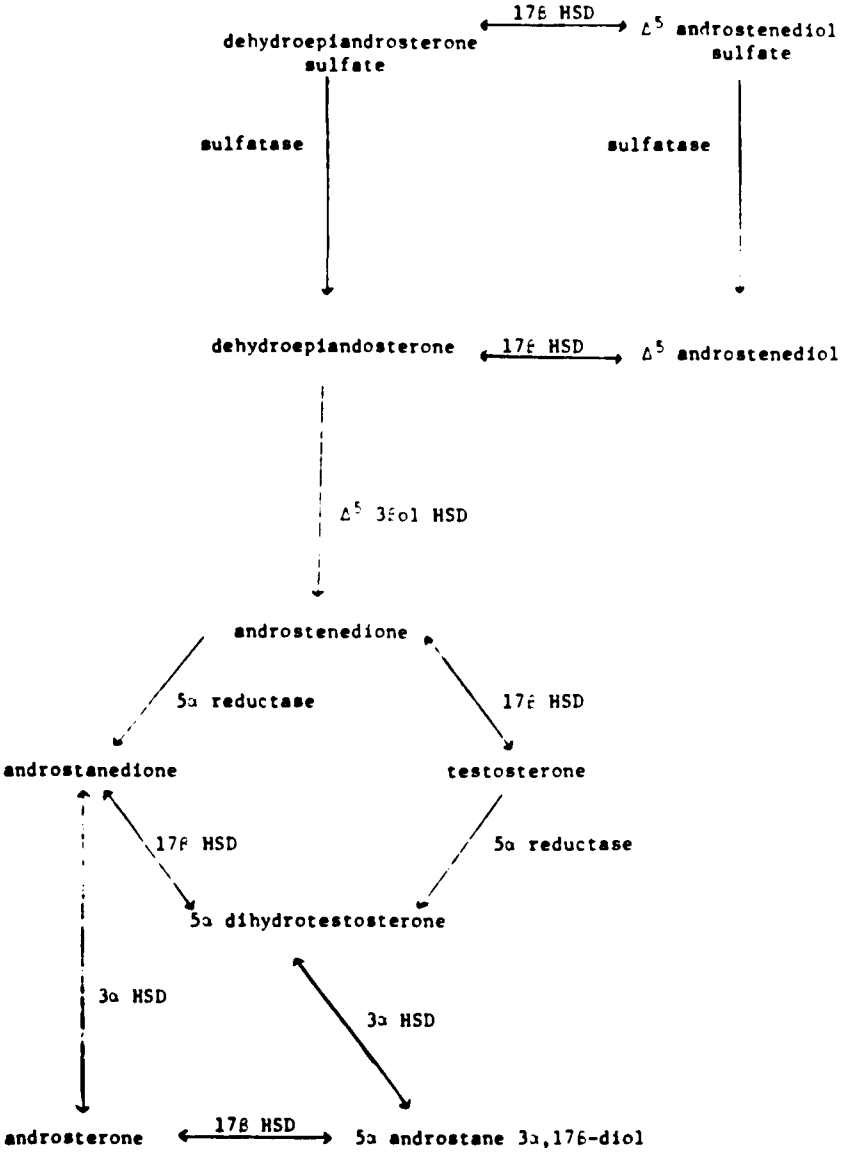
Having chosen the technique of superfusion with two isotopically labeled steroids for the reasons given above, the metabolism of

several pairs of metabolically related C_{19} steroids was studied by using endometrial tissue slices. Figure 1 shows pertinent metabolic relationships,

The metabolic chart begins with dehydroepiandrosterone sulfate, the major circulating C_{19} compound in human plasma. It can be hydrolyzed to dehydroepiandrosterone by a steroid alcohol sulfatase or might be reduced to Δ^5-androstenediol sulfate which can be hydrolyzed to Δ^5-androstenediol. Dehydroepiandrosterone can be converted to androstenedione by $\Delta^5 3\beta$-ol dehydrogenase or be reduced to Δ^5- androstenediol. Androstenedione is reduced to testosterone by 17β hydroxysteroid dehydrogenase or to androstanedione by 5α reductase. Testosterone is reduced to 5α dihydrotestosterone by 5α reductase or oxidized to androstenedione by 17β hydroxysteroid dehydrogenase. The 5α dihydrotestosterone is oxidized to androstanedione by 17β hydroxysteroid dehydrogenase or reduced by 3α or 3β hydroxysteroid dehydrogenase to 3α or 3β,5α,17β-androstanediol. The androstanedione is reduced by 3α hydroxysteroid dehydrogenase to androsterone which can be reduced further to 3α or 3β,5α,17β-androstanediol.

With this pattern in mind, specimens of proliferative and secretory endometrium were superfused with mixtures of dehydroepiandrosterone sulfate and dehydroepiandrosterone, mixtures of testosterone and androstenedione, mixtures of testosterone and 5α dihydrotestosterone and mixtures of androstenedione and androstanedione. The metabolites isolated and examined during these experiments were dehydroepiandrosterone, testosterone, androstenedione, 5α dihydrotestosterone, androstanedione and androsterone. The data obtained from

Figure 1


17β HSD
dehydroepiandrosterone sulfate
Δ5 androstenediol sulfate
sulfatase
sulfatase
dehydroepiandosterone
17β HSD
Δ5 androstenediol
Δ5 3βol HSD
androstenedione
5α reductase
17β HSD
androstanedione
testosterone
17β HSD
5α reductase
5α dihydrotestosterone
3α HSD
3α HSD
androsterone
17β HSD
5α androstane 3α,17β-diol

this combination of experiments will be used to discuss the metabolism of androgens by normal human endometrium. Finally, several batch incubation experiments were carried out with testosterone and endometrial adenocarcinoma cells in order to compare their metabolism with that of normal endometrium.

5. General Methodology

a. Tissue Preparation

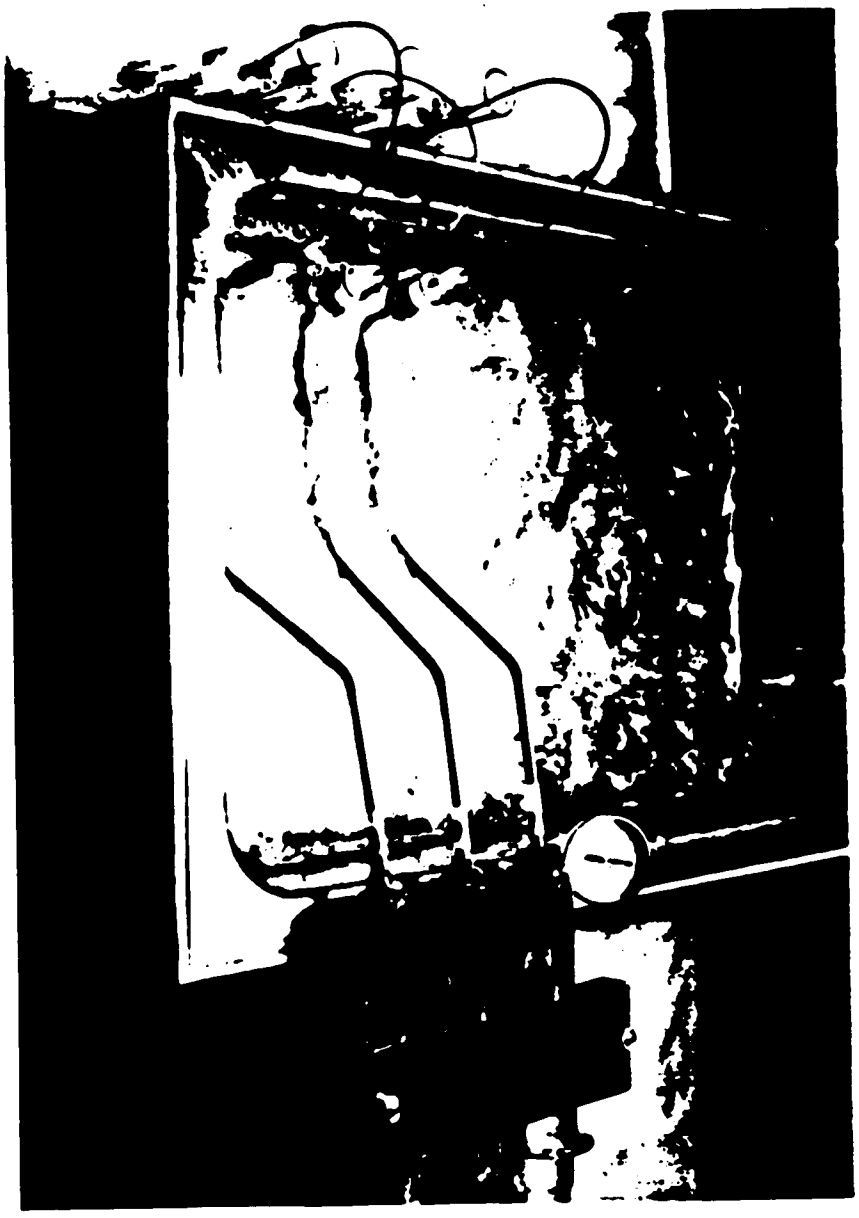
Endometrial tissue from curettings or removed from uteri at hysterectomy was brought immediately to the laboratory from the operating room in cold saline. Blood clots and mucus were removed from the tissue which was then sliced to a 200 μg thickness by a TC-2 tissue sectioner (Sorvall, Inc.) or minced with scissors. The tissue was washed free of debris with cold saline, dried with absorbant Miracloth (Chicopee, Mills Inc., N.Y.) and weighed. Between 50 to 400 mg of tissue was placed in the superfusion chamber of the perfusion apparatus which is shown in Figure 2. An additional aliquot of tissue from each specimen was frozen and saved for determination of protein content according to the Lowry method (113). Small samples also were fixed in 10% neutral buffered formalin for histologic examination.

b. Superfusion Procedure

The superfusion experiments were carried out at 37 C for two hours usually at a flow rate of 15 ml/hr. The superfusate was collected in ice-cooled test tubes in two half hour aliquots for the first hour and three twenty minute aliquots for the second hour. Samples of the last hour's aliquots were mixed with 500 μg of the appropriate steroid carriers in order to correct for recovery.

At the conclusion of the superfusion, the tissue slices were transferred to a Miracloth filter mounted on a Millipore filter holder attached to a suction flask. The slices were washed rapidly 5 times with a total of 15 ml of cold saline. In four of the super-

Figure 2



fusion experiments, aliquots of each of the five washes were counted in order to determine that all extracellular contaminating medium is removed by five washes. It is important to be assured that the $^{3}H/^{14}C$ ratios of the superfused compounds that are extracted from the tissue correspond to the intracellular isotopic ratios of these compounds. Table 2 shows results from one of these experiments in which the $^{3}H/^{14}C$ ratio in the compounds removed from the tissue during the last wash is about equal to the corresponding ratio of the compound remaining in the tissue. The tissue was then homogenized with 500 µg of the same carriers as had been added to the superfusate.

c. Tracers

All the tracers utilized in these experiments were purchased from New England Nuclear Corp., Boston. Their specific activities are given in the individual sections on materials and methods for each group of superfusion experiments. Their radiochemical purity was determined in one of three ways. One, by thin layer chromatography (TLC) on pre-coated silica gel plates (E. M. Merck) after addition of chemically pure standards supplied by Steraloids. The observance of a single peak of radioactivity on a radiochromatogram indicated purity. Two, by crystallization after the addition of chemically pure carrier. Identity of the specific activity of the mixture before crystallization with the specific activity in the crystals and the mother liquor indicates purity. Three, by mixing a tritiated compound with its corresponding radiochemically pure ^{14}C-labeled steroid, adding chemically pure carrier and crystallizing the mixture either with or without prior thin layer chromatography.

Table 2

Evidence that Tissue is Washed clear of Buffer with Five Washes

Compound	$^{3}H/^{14}C$ Ratios		
	Perfused	Wash 1	Wash 5	Washed tissue
Testosterone	940	115	104
	29		
Androstenedione	8.6	19	23

Identity of the $^{3}H/^{14}C$ ratio in the precrystallization mixture, the crystals and the mother liquor indicates radiochemical homogeneity. The purity of the isotopes was checked immediately preceding each experiment and they were purified when necessary by thin layer chromatography.

All measurements of radioactivity were performed with a liquid scintillation spectrometer (Isocap 300, Nuclear Chicago Corp.) using Scintiverse (Fisher Scientific Co.) as scintillation fluid. The counting efficiencies were about 33.5% for ^{3}H and 65.7% for ^{14}C.

d. Superfusion medium

The medium used for the superfusions was Gey's balanced salt solution (GBSS) obtained from Grand Island Biological Co. Immediately preceding the perfusion, a mixture of 95% O_2 and 5% CO_2 was bubbled into the medium for 10 minutes. The steroid tracers at concentrations indicated for each experiment were dissolved in methanol and added to the buffer with a final concentration of less than 2% methanol. Before and after each superfusion, aliquots of medium were removed from the syringe to determine the exact concentration of tracers in the buffer.

e. Isolation of Steroids

The labeled steroids were extracted from the tissue and superfusate with organic solvents and isolated on pre-coated silica gel plates using thin layer chromatography. The solvent systems are listed in Table 3. Following TLC, the steroids were purified by high pressure liquid chromatography (HPLC) (Waters Assoc., Bedford, Mass.). The HPLC solvent systems are also listed in Table 3. The

Table 3

Chromatography Systems

Designation	Support	Solvent System (V/V)	Compound	Relative mobility
Thin layer chromatography				
TLC-1	Merck GF-254	Chloroform: acetone: hexane 4:1:3	5α-androstane-3α,17β-diol	0.15
			5-androstene-3α,17β-diol	0.20
			T	0.30
			D	0.43
			DHT	0.50
			androsterone	0.50
			Δ	0.60
			androstanedione	0.70
TLC-2	Merck GF-254	Benzene: acetone: methanol 35:10:10	5-androstene-3β,17β-diol 3-sulfate	0.20
			DS	0.26
			D	0.86
TLC-3	Merck GF-254	Chloroform: ethyl acetate 4:1	5-androstene-3β,17β-diol	0.20
High pressure liquid chromatography				
HPLC-1	μ-Porasil	Chloroform: isooctane 3:2	androstanedione	0.63
			Δ	1.0
			D	1.1
			DHT	1.3
			androsterone	1.7
			T	2.7
			androstane-3β,17β-diol	3.1
			androstane-3α,17β-diol	3.6
HPLC-2	Micro-bondapak C_{18}	Acetonitrile: water 1:1	androstane-3β 17β-diol	0.5
			androstane-3α,17β-diol	1.0

amount of carrier recovered after purification was measured by U.V. spectrometry (Beckman Instruments, Mountainside, N.J.) or by differential refractometry. Following quantitation, the radiochemical purity of the recovered steroids was determined by crystallization to constant specific activity after the addition of approximately 5 mg of authentic standards.

f. Criteria for Purity of Isolated Steroids

The criteria for purity of the metabolic products of the perfused labeled precursors was the establishment of their constant specific activities and consistent $^{3}H/^{14}C$ ratios after repeated purification. Following perfusion, the metabolites were first separated by thin layer chromatography. The testosterone and androstenedione isolated after the TLC were quantitated by U.V. spectrometry and aliquots were counted to determine their specific activity. The testosterone and androstenedione were then further purified by HPLC and their specific activities again determined, so that comparisons of the specific activities from the two purifications could be made. Their consistancy is indicative of their chemical purity. The metabolites which are not U.V. absorbing were purified after TLC by HPLC and were quantitated by differential refractometry. Aliquots of these were counted also to determine their specific activity.

Following the purification and quantitation, 4 or 5 mg of additional chemically pure carriers were added in order to crystallize the steroids and determine their specific activity. Knowing the amount of initial carrier remaining plus the carrier added for crystallization, it is possible to take both into account to determine

the specific activity. Three crystallizations were usually performed. Any impurity would appear in the mother liquor resulting in a higher specific activity than the crystals and the percent of impurity could be calculated as follows:

$$\left(1 - \frac{\text{sp. act final crystals}}{\text{sp. act. initial mixture}}\right) \times 100$$

6. Calculations of Kinetic Parameters: Theory

a. Definitions

The experiments presented here involve the perfusion of endometrial tissue with two metabolically related tracers. For the purpose of discussing the formulae used in calculating the various parameters of these experiments, one tracer will be designated ^{3}H-A and the other ^{14}C-B. Referring to Fig. 3A which pictorically represents the pattern of perfusion of these two tracers, we will consider the following fractional rates.

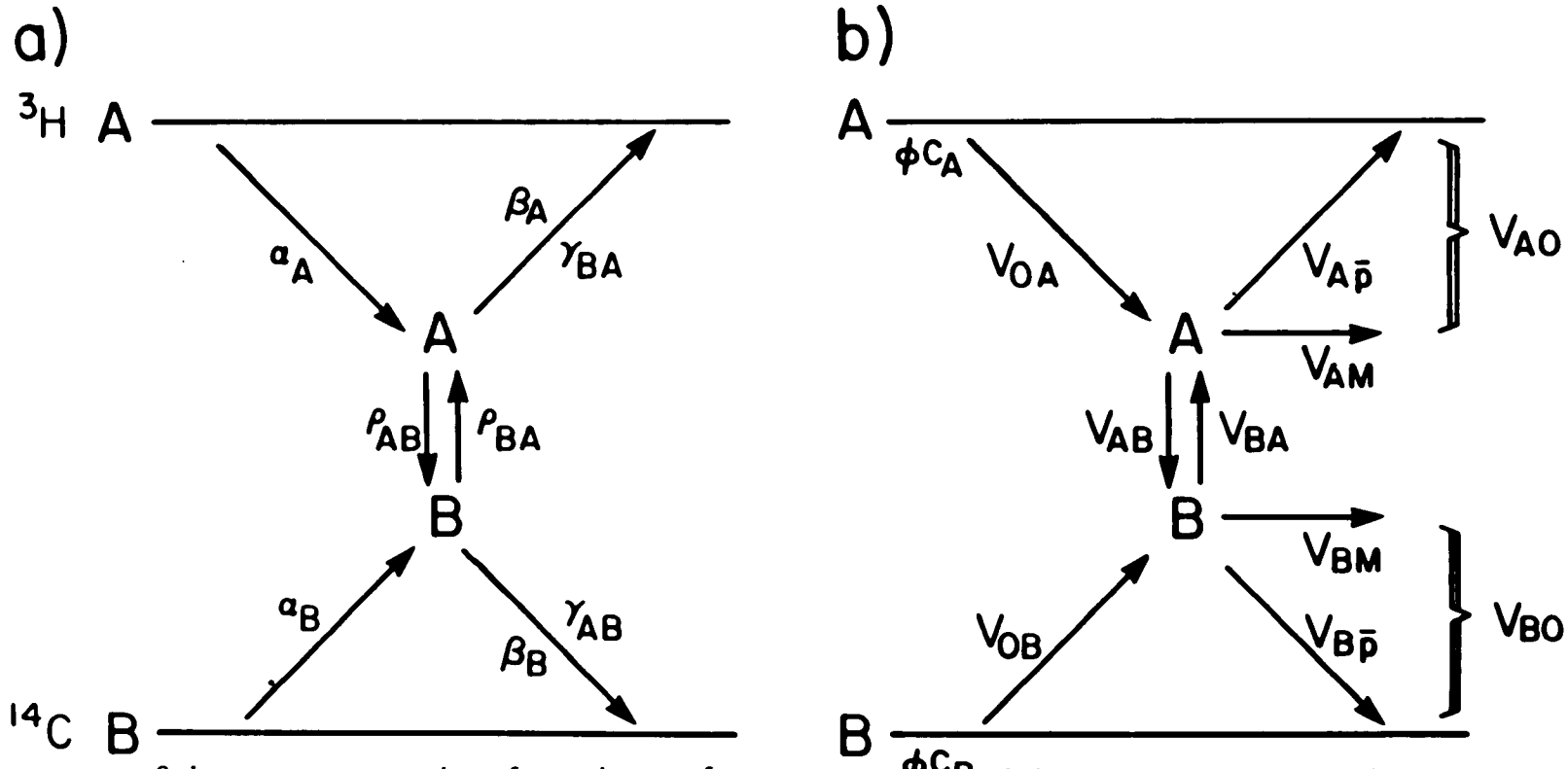
- The fraction of the perfused tracer which enters the tissue, α.
- The fraction of the perfused tracer which is released back to the perfusate, β.
- The fraction of the perfused tracer which is released by the tissue as a metabolite, γ.
- The fraction of each tracer entering the tissue which is converted to its co-perfused compound in the tissue, ρ.

b. Fractional rates

(i) Entry (α)

Alpha represents the fraction of the perfused tracer ^{3}H-A which enters the tissue. The fraction of the tracer which by-passes the tissue is $1-\alpha_A$. The concentration of ^{3}H-A in the perfusion buffer is c_A^{3H}. The total concentration of ^{3}H-A in the perfusate ($c_{A_p}^{3H}$) is the sum of that tracer which entered the tissue and was released, ($c_{A_p}^{3H}$)$'$ plus the tracer which by-passed the tissue ($c_{A_p}^{3H}$)$''$, i.e.

Figure 3.



Scheme representing fractions of perfused tracers A and B which enter the tissue are interconverted and released from the tissue to the medium

Scheme representing the rates of synthesis interconversion and removal of the compounds A and B in the tissue

$$c^{^3H}_{A\bar{P}} = \left(c^{^3H}_{A\bar{P}}\right)' + \left(c^{^3H}_{A\bar{P}}\right)'' \qquad (1)$$

It follows from the definition of $(c^{^3H}_{A_{\bar{P}}})''$ that

$$1 - \alpha_A = \frac{\left(c^{^3H}_{A\bar{P}}\right)''}{c^{^3H}_{A}} \qquad (2)$$

To estimate α, it is necessary to separate these two components of the tracer in the perfusate. This is done by perfusing the tissue with tracer ^{3}H-A and with another tracer which is enzymatically interchangeable in the tissue with compound A, i.e. ^{14}C-B. Thus, the A which enters the tissue and is released will have a double label (^{3}H and ^{14}C), while the compound A which by-passed the tissue has a single label (^{3}H). At the isotopic steady state during superfusion, the ^{3}H/^{14}C ratio of compound A in the perfusate can be expected to have the same ^{3}H/^{14}C ratio as compound A in the tissue. This has been experimentally verified by multiple washings with buffer of the perfused tissue with the eventual result that the compound extracted from the final washing has a ^{3}H/^{14}C ratio equivalent to that of the same compound extracted from the washed tissue.

Thus,

$$\frac{\left(c^{^3H}_{A\bar{P}}\right)'}{c^{^{14}C}_{A\bar{P}}} = \left(\frac{^3H}{^{14}C}\right)_{At} \qquad (3)$$

where $(^3H/^{14}C)_{At}$ is the isotopic ratio of A in the tissue. This equation can be rearranged to:

$$\left(C_{A\bar{P}}^{3H}\right)' = \left(\frac{^3H}{^{14}C}\right)_{At} C_{A\bar{P}}^{14C} \qquad (4)$$

Therefore, by knowing the concentration of ^{14}C-A in the perfusate ($C_{A_{\bar{P}}}^{14C}$) and the $^3H/^{14}C$ ratio of A in the tissue, one is able to determine the fraction of ^3H-A which has entered and been released by the tissue. One can see from equations 1, 2 and 4 that:

$$1-\alpha_A = \frac{\left(C_{A\bar{P}}^{3H}\right)''}{C_A^{3H}} = \frac{C_{A\bar{P}}^{3H} - \left(C_{A\bar{P}}^{3H}\right)'}{C_A^{3H}} \qquad (5)$$

and

$$1-\alpha_A = \frac{C_{A\bar{P}}^{3H} - \left(^3H/^{14}C\right)_{At} C_{A\bar{P}}^{14C}}{C_A^{3H}} \qquad (6)$$

Rearranging this latter equation, one can calculate α_A as:

$$\alpha_A = 1 - \frac{C_{A\bar{P}}^{3H} - \left(^3H/^{14}C\right)_{At} C_{A\bar{P}}^{14C}}{C_A^{3H}} \qquad (7)$$

When there is enzymatic interconvertibility between compound A and B, then due to symmetry:

$$\alpha_B = 1 - \frac{C_{B\bar{P}}^{14C} - \left(^{14}C/^3H\right)_{Bt} C_{B\bar{P}}^{3H}}{C_B^{14C}} \qquad (8)$$

(ii) Exit (β)

The fraction of the perfused tracer ^{3}H-A which is released to the perfusate is called β_A. Then,

$$\beta_A = \frac{\left(C_{A\bar{P}}^{^3H}\right)'}{C_A^{^3H}} \qquad (9)$$

and from equation 4,

$$\beta_A = \frac{\left(^3H/^{14}C\right)_{A_f} C_{A\bar{P}}^{^{14}C}}{C_A^{^3H}} \qquad (10)$$

β_B is defined in the same fashion,

$$\beta_B = \frac{\left(^3H/^{14}C\right)_{B_f} C_{B\bar{P}}^{^3H}}{C_B^{^{14}C}} \qquad (11)$$

The expression β/α used in discussing some of these experiments is the fraction of the perfused tracer which enters the tissue and is released unmetabolized to the perfusate.

(iii) Metabolism and Exit (γ)

The fraction of the superfused tracer ^{3}H-A which is released by the tissue as compound B (γ_{AB}) and the fraction of the superfused tracer ^{14}C-B which is released by the tissue as compound A (γ_{BA}) are the ratios of the concentrations of the labeled compounds in the perfusate and the concentrations of the precursors in the perfusion medium, i.e.

$$\gamma_{AB} = \frac{c_{B\bar{P}}^{^3H}}{c_A^{^3H}} \qquad (12)$$

and

$$\gamma_{BA} = \frac{c_{A\bar{P}}^{^{14}C}}{c_B^{^{14}C}} \qquad (13)$$

The fraction of the perfused tracer ^{3}H-A which enters the tissue and is released to the medium as ^{3}H-B is γ_{AB}/α_A; γ_{BA}/α_B is the fraction of the perfused tracer ^{14}C-B which enters the tissue and is released to the medium as ^{14}C-A.

(iv) Metabolic Conversions (ρ)

The conversion or transfer factor, ρ, is defined as that fraction of the superfused tracer which is converted in the tissue to its co-perfused compound, i.e. the fraction of ^{3}H-A entering the tissue which is converted to compound B (ρ_{AB}) and the fraction of ^{14}C-B entering the tissue which is converted to compound A (ρ_{BA}).

A value in terms of isotopic data can be given to that factor, e.g. ρ_{AB}, because it follows from its definition that the ^{3}H/^{14}C ratio in compound B in the tissue is determined by the ratio of the rates at which compound B labeled with ^{3}H and ^{14}C appears <u>de novo</u> in the tissue. For the ^{3}H label this rate is given by the amount of superfused ^{3}H-A which enters the tissue per unit of time ($\phi c_A^{^3H} \alpha_A$) and the fraction of that amount which is converted to compound B (ρ_{AB}).

The symbol ϕ denotes the rate at which the medium is superfused (ml/hr). For the ^{14}C label, it is the amount of perfused ^{14}C-B which enters the tissue per unit of time ($\phi_B {}^{14}C_{\alpha_B}$). That is,

$$\left(\frac{^3H}{^{14}C}\right)_{Bt} = \frac{\phi\, C_A^{3H}\, \alpha_A\, \rho_{AB}}{\phi\, C_B^{Mo}\, \alpha_B} \qquad (14)$$

This equation can be arranged to:

$$\rho_{AB} = \frac{\alpha_B}{\alpha_A} \frac{\left(\frac{^3H}{^{14}C}\right)_{Bt}}{\left(\frac{^3H}{^{14}C}\right)_{superfused}} \qquad (15)$$

Because of the symmetry of this system,

$$\rho_{BA} = \frac{\alpha_A}{\alpha_B} \frac{\left(\frac{^3H}{^{14}C}\right)_{superfused}}{\left(\frac{^3H}{^{14}C}\right)_{At}} \qquad (16)$$

c. Rates

(i) Entry (v_{0A} and v_{0B})

The above described parameters of the superfusion experiments can be used for the estimation of rates. In Fig. 3b, where v_{0A} and v_{0B} are the rates of entry into the tissue of the superfused tracers ^3H-A and ^{14}C-B. These rates are determined by the α's of the tracer, the concentration of the tracer in the medium and the flow rate of the medium.

$$v_{0A} = \phi\, C_A^{3H}\, \alpha_A \qquad (17)$$

and

$$v_{OB} = \phi c_B^{1\%} \alpha_B \qquad (18)$$

(ii) Production Rates (PR_A and PR_B)

The production rate is defined as the rate at which the compound appears de novo in the tissue. In endometrial tissue which does not synthesize steroids, the only source for A and B would be the superfused A and B. v_{OA} and v_{OB} are part of the production rates (PR_A, PR_B) of the exogenous superfused compounds in the tissue. The production rate also includes that fraction of the co-superfused compound which is converted within the tissue, i.e.

$$PR_A = v_{OA} + \rho_{BA} v_{OB} \qquad (19)$$

and

$$PR_B = v_{OB} + \rho_{AB} v_{OA} \qquad (20)$$

It is possible to determine the production rates in terms of the isotopic data. For instance, the specific activity of ^{3}H-A in the tissue ($a_{At}^{^3H}$) is equal to the ratio of the rate of the de novo appearance of ^{3}H-A in the tissue and the rate at which compound A enters the tissue de novo, viz.

$$a_{At}^{^3H} = \frac{\phi c_A^{^3H} \alpha_A}{PR_A} \qquad (21)$$

By definition,

$$a^{3H}_{At} = \frac{c^{3H}_{At}}{c_{At}} \qquad (22)$$

where c^{3H}_{At} (cpm/g) is the concentration of radioactivity present as compound A in the tissue and c_{At} (pmol/g) is the concentration of compound A in the tissue. When the only source of A and B in the tissue is superfused A and B, as is the case in these studies with human endometrium, c_{At} can be estimated from steady state isotopic data. Since c_{At} equals the sum of compound A derived from compounds A and B superfused (supf), it follows that

$$c_{At} = \frac{c^{3H}_{At}}{a^{3H}_{A\,supf}} + \frac{c^{14C}_{At}}{a^{14C}_{B\,supf}} \qquad (23)$$

Therefore, the production rate of compound A in the tissue can be determined solely from the isotopic data as shown by equations 21, 22 and 23. Thus,

$$PR_A = \frac{\phi c^{3H}_A \alpha_A}{a^{3H}_{At}} = \phi \alpha_A \frac{c^{3H}_A}{c^{3H}_{At}} \left(\frac{c^{3H}_{At}}{a^{3H}_{A\,supf}} + \frac{c^{14C}_{At}}{a^{14C}_{B\,supf}} \right) \qquad (24)$$

The equivalent equation for the production rate of compound B is:

$$PR_B = \phi \alpha_B \frac{c^{14C}_B}{c^{14C}_B} \left(\frac{c^{14C}_{Bt}}{a^{14C}_{B\,supf}} + \frac{c^{3H}_{Bt}}{a^{3H}_{A\,supf}} \right) \qquad (25)$$

(iii) Metabolic Conversion (v_{AB}, v_{BA})

These rates can be calculated from the PRs and ρs. On Fig. 3b, the rate v_{AB} in a steady state is the sum of the rates at which A is converted to B for the first time ($\rho_{AB}PR_A$), for the second time ($\rho_{AB}PR_A \times \rho_{BA}\rho_{AB}$), for the third time ($\rho_{AB}PR_A \times \rho_{BA}\rho_{AB} \times \rho_{BA}\rho_{AB}$) and so on. Then,

$$v_{AB} = \rho_{AB} PR_A + \rho_{AB} PR_A \rho_{AB} \rho_{BA} + \rho_{AB} PR_A (\rho_{BA} \rho_{AB})^2 + \ldots.$$

$$v_{AB} = \rho_{AB} PR_A (1 + \rho_{AB}\rho_{BA} + \rho_{AB}^2 \rho_{BA}^2 + \ldots.)$$

or

$$v_{AB} = \frac{\rho_{AB} PR_B}{1 - \rho_{AB} \rho_{BA}} \qquad (26)$$

Similarily,

$$v_{BA} = \frac{\rho_{BA} PR_B}{1 - \rho_{AB} \rho_{BA}} \qquad (27)$$

(iv) Exit (v_{AO}, v_{BO})

The remaining rates to be discussed are those relating to the perfused compounds leaving the tissue v_{AO} and v_{BO}. In a steady state:

$$v_{AO} = v_{OA} + v_{BA} - v_{AB} \qquad (28)$$

and

$$v_{BO} = v_{OB} + v_{AB} - v_{BA} \qquad (29)$$

As shown in Fig. 3b,

$$v_{Ao} = v_{A\bar{p}} + v_{AM}, \tag{30}$$

that is, v_{OA} is the sum of the rates at which A is released to the medium ($v_{A_{\bar{p}}}$) or is converted to metabolites (M) by pathways not involving B as an intermediate. The same can be said for the release of compound B, viz.

$$v_{Bo} = v_{B\bar{p}} + v_{BM} \tag{31}$$

The rate of exit of compound A from the tissue ($v_{A_{\bar{p}}}$) equals the fraction of the concentration of compound A perfused (c_A) which is released unmetabolized ($\phi c_A \beta_A$) plus the fraction of compound B superfused which enters the tissue and is released as compound A ($\phi c_B \gamma_{BA}$). That is:

$$v_{A\bar{p}} = \phi(c_A \beta_A + c_B \gamma_{BA}) \tag{32}$$

and for compound B:

$$v_{B\bar{p}} = \phi(c_B \beta_B + c_A \gamma_{AB}) \tag{33}$$

The rates v_{AM} and v_{BM} can then be calculated as follows:

$$v_{AM} = v_{Ao} - v_{A\bar{p}} \tag{34}$$

and

$$v_{BM} = v_{Bo} - v_{B\bar{p}} \tag{35}$$

II. EXPERIMENTAL

1. Conversion of Dehydroepiandrosterone Sulfate to Dehydroepiandrosterone

a. Introduction

There is evidence for the hydrolysis of sulfated estrogens by human and mouse mammary tumors (115), MCF-7 human breast cancer cells in culture (116), ovine pituitary and hypothalamic tissue minces (117) and rat and human endometrial tissue minces (118,119). Studies of human breast tumors have shown that these tumors also hydrolyze dehydroepiandrosterone sulfate (115,120,121). Therefore, it was expected that dehydroepiandrosterone sulfate would be hydrolyzed by endometrial tissue. The physiologic significance of this hydrolysis is questionable, however, since it is unclear whether sulfated steroids pass through capillary walls as easily as the unconjugated compounds. The rabbit uterus did not take up sulfated estrone which had been injected intraaortically (122). However, one hour after the injection of ^{3}H-estradiol in ovariectomized C3H mice, 50 to 70% of the radioactivity in the mammary tumor cytosol was due to estrogen conjugates, mostly sulfates, which were presumed to be of plasma origin (114).

b. Material and Methods

Six similar superfusion experiments were performed using the pair of [7-^{3}H] dehydroepiandrosterone sulfate (DS) (S.A. = 24 Ci/mmol) and [4-^{14}C] dehydroepiandrosterone (D) (S.A. = 57.5 mCi/mmol). The concentrations of tracers in the superfusion buffer (GBSS) were approximately 2.0 x 10^{6} cpm/ml (112 pmol/ml) of ^{3}H-DS and 68,000 cpm/ml (810 pmol/ml) of ^{14}C-D with the resultant $^{3}H/^{14}C$ ratio of 29. In one of

these experiments, the concentrations were approximately 600,000 cpm/ml of ^{3}H-DS and 17,000 cpm/ ml of ^{14}C-D.

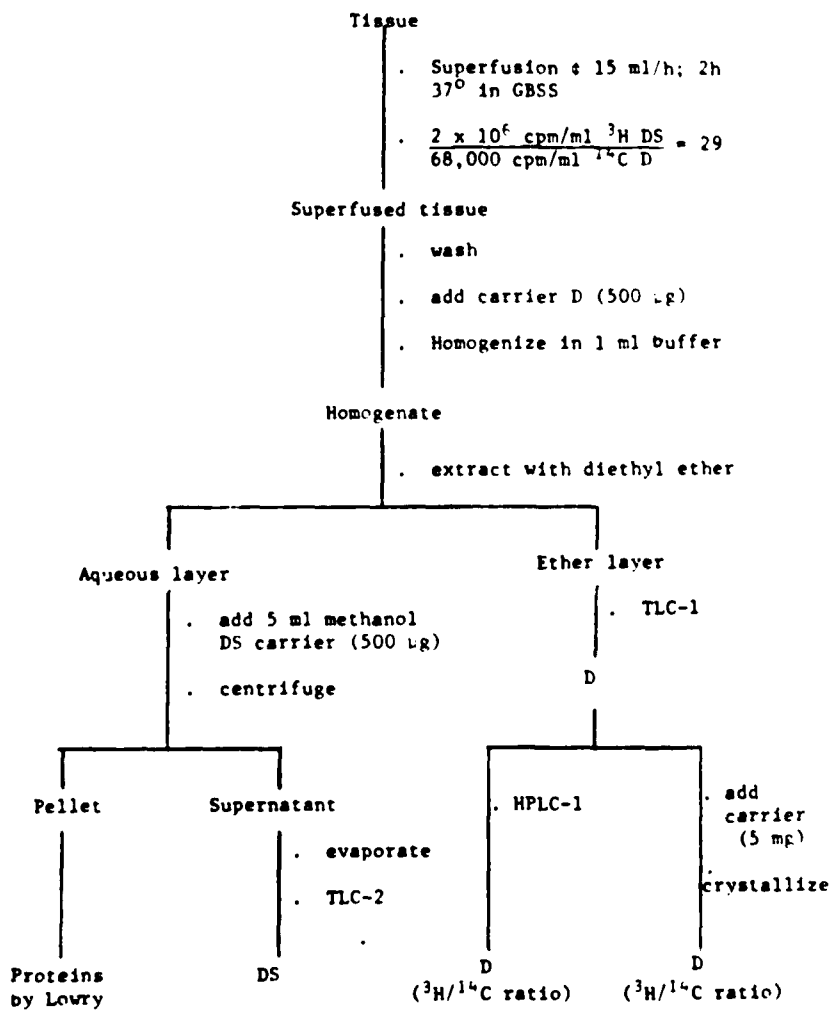
The tissue used in these six experiments was derived from three proliferative endometria and three secretory endometria and was prepared for superfusion in GBSS as previously described in the section on general materials and methods. Five of the superfusions lasted for two hours with four at a flow rate of 15 ml/hr and one at 20 ml/hr. The sixth experiment with the lowest concentration of tracers was a superfusion lasting one hour at a flow rate of 10 ml/hr because of an insufficient amount of perfusion medium.

The experimental design used for the D and DS superfusion experiments is shown in Flow Chart 1. The tissue slices were washed 5 times with cold saline and homogenized with 500 µg of carrier for D in 1 ml of buffer. Following homogenization, the tissue was extracted twice with 5 ml diethyl ether and the aqueous layer was frozen with dry ice in methanol and the ether layer was decanted. The ether extract, washed with 1 ml of water and evaporated under nitrogen, was chromatographed using the system TLC-1. Radioactivity zones were located with a radioscanner (Packard Instruments). The carrier zone was identified after spraying a narrow longitudinal strip on the plate with a mixture of 5% H_2SO_4 and ethanol and eluted with ethyl acetate.

The eluted sample of D was divided in half. One half was chromatographed using the system HPLC-1. The other half was mixed with 5 mg of D and crystallized three times from methanol-water. Aliquots of the purified D were counted after the HPLC and after crystallization to determine the $^{3}H/^{14}C$ ratio of the compound.

Flow Chart 1

Experimental Design; Dehydroepiandosterone Sulfate and Dehydroepiandrosterone Superfusion Experiments



In 5 of the 6 experiments, the aqueous layer of the homogenate remaining after ether extraction was mixed with 5 ml of methanol and 500 μg of DS carrier. The precipitated protein was separated by centrifugation and saved for protein determination. The supernatant was evaporated and chromatographed on the system TLC-2. The DS band was eluted twice with 4 ml of methanol to measure $^{3}H/^{14}C$ ratios.

c. Results

The fraction of the ^{3}H dehydroepiandrosterone sulfate converted to dehydroepiandrosterone in the perfusion experiments was determined by using formula 15, which defines the fraction of the ^{3}H compound tracer A which is converted to ^{3}H compound B in the tissue or in this case, the fraction of ^{3}H-dehydroepiandrosterone sulfate which is converted to ^{3}H-dehydroepiandrosterone. For the purpose of calculation in these experiments, the assumption is made that the ratio of α_A to α_B in this formula is 1, which means that the same fraction of each compound interacts with the cells. This assumption is made with the recognition that it still is undetermined that sulfated steroids enter cells. If hydrolysis occurs in the plasma membrane, the ^{3}H-labeled free steroid formed from DS may have the same fate as ^{14}C-D interacting with the cell and the calculations would be valid. The fraction of dehydroepiandrosterone sulfate hydrolyzed to dehydroepiandrosterone ($\rho_{DS,D}$) is estimated by dividing the ratio of the $^{3}H/^{14}C$ of dehydroepiandrosterone in the tissue by the $^{3}H/^{14}C$ ratio of the superfused mixture of dehydroepiandrosterone sulfate and dehydroepiandrosterone. The value of the transfer factor in these six experiments, half with proliferative tissue and half with secretory tissue,

is shown in Table 4. The isotope ratios of the dehydroepiandrosterone sulfate and dehydroepiandrosterone perfused are listed also in this table. It can be seen that the $\rho_{DS,D}$ range from 10 to 20% with no distinction due to menstrual cycle phase.

The radiochemical purity of this dehydroepiandrosterone isolated from the tissue was determined by crystallization following purification by TLC and HPLC. Crystallization data can be found in Table 5.

In conclusion, when perfusing endometrial tissue with ^{3}H-dehydroepiandrosterone sulfate and ^{14}C dehydroepiandrosterone, there is a 10 to 20% hydrolysis of the sulfate, regardless of the phase of the menstrual cycle.

d. Discussion

Assuming that sulfated compounds such as dehydroepiandrosterone sulfate pass through capillary walls as found by Vignon et al (116) in C3H mice and assuming that endometrial tissue concentrated dehydroepiandrosterone almost twenty-fold, as found by Guerrero et al (106), there is a potential pool of dehydroepiandrosterone in endometrial tissue which can be converted to androstenedione by $\Delta^{5}3\beta$-ol dehydrogenase. With plasma concentrations in women of dehydroepiandrosterone sulfate of around 2 μg/ml and dehydroepiandrosterone around 5 ng/ml, the size of this substrate pool with a conversion rate of DS to D of 20% would be considerable. The androstenedione from dehydroepiandrosterone then would be available for the 17β hydroxysteroid dehydrogenase reduction to testosterone, which can be 5α reduced to dihydrotestosterone by endometrial tissue, thus producing a physiologically active androgen which may influence endometrial growth.

Table 4

Experimental Results: Dehydroepiandrosterone Sulfate and Dehydroepiandrosterone Superfusion Experiments

Experiment	Endometrial specimen	Concentration of tracers in perfused medium $\frac{^3H\ DS\ cpm/ml\ (pmol/ml)}{^{14}C\ D\ cpm/ml\ (pmol/ml)}$	Tissue $^3H_{DS}/^{14}D$ (cpm/cpm)	Conversion factor DS→D (%)
S25	M.P.	$\frac{2.0 \times 10^6 (123)}{68,000\ (809} = 29$	5.8	19
A 17	L.P.	$\frac{2.0 \times 10^6 (123)}{74,000\ (881)} = 27$	4.8	16
J5	S-18	$\frac{600,000\ (33.6)}{17,000\ (202)} = 35$	5.0	12
N6	S-20	$\frac{1.9 \times 10^6 (106)}{64,000\ (762)} = 30$	6.0	20
J31	S-21	$\frac{2.0 \times 10^6 (123)}{70,000\ (833)} = 29$	4.1	14
D21	Proliferative with hyperplasia	$\frac{2.2 \times 10^6 (123)}{68,000\ (809)} = 32$	4.1	10

M.P., mid-proliferative endometrium; L.P., late proliferative endometrium; S-number, secretory endometrium and day of the menstrual cycle.

Table 5

Isotopic Data from Crystallization of Dehydroepiandrosterone Isolated from Tissue

Exp. #	Sample	Parameter			
		$^3H/^{14}C$	Specific activities (cpm/mg) ^3H	^{14}C	Weight of crystals (mg)
S25	last chromatography	5.8			
	1st mother liquor	6.9	9000	1300	0.2
	1st crystallization	6.0	12,000	2000	1.0
	2nd crystallization	5.4	7000	1300	1.0
	3rd crystallization	5.8	7500	1300	0.9
A17	last chromatography	4.8			
	1st mother liquor	7.0	7000	1000	0.2
	2nd mother liquor	4.7	7000	1500	0.2
	1st crystallization	4.7	7000	1500	1.0
	2nd crystallization	4.8	7200	1500	1.1
	3rd crystallization	4.8	7200	1500	1.0
J5	last chromatography	5.0			
	1st mother liquor	4.6	2300	500	0.1
	2nd mother liquor	4.9	2200	450	0.1
	1st crystallization	4.8	2900	600	1.1
	2nd crystallization	5.0	3000	600	1.0
	3rd crystallization	5.0	3000	600	1.6
N6	last chromatography	5.7			
	1st mother liquor	5.0	20,000	4000	0.2
	1st crystallization	6.0	27,000	4500	1.0
	2nd crystallization	6.0	18,000	3000	1.1
	3rd crystallization	6.0	27,000	4500	1.7
J31	last chromatography	3.8			
	1st mother liquor	4.0	10,500	2600	0.1
	3rd mother liquor	3.5	7000	2000	0.1
	1st crystallization	4.0	9700	2400	1.0
	2nd crystallization	4.0	9500	2400	1.2
	3rd crystallization	4.1	9500	2300	1.8
D21	last chromatography	4.0			
	1st mother liquor	4.5	21,000	4700	0.2
	1st crystallization	4.3	20,000	4600	1.1
	2nd crystallization	4.1	19,000	4600	0.8

2. Interconversion of Testosterone and Androstenedione

a. Introduction

Testosterone and androstenedione are interconvertible by the action of 17β hydroxysteroid dehydrogenase and both can be 5α reduced to dihydrotestosterone and androstanedione, respectively. The dihydrotestosterone and androstanedione in turn also are interconvertible by 17β hydroxysteroid dehydrogenase. Thus, the cellular concentrations of biologically active dihydrotestosterone in endometrial tissue can be controlled in part by this enzyme. Since it has been determined that levels of activity of 17β hydroxysteroid dehydrogenase in the endometrium vary during the menstrual cycle, being higher in secretory tissue, and since this enzyme can use androgens as substrates although it prefers estrogens (123), it was of interest to determine whether the changes in the level of enzyme activity would be reflected by changes in the metabolism of testosterone and androstenedione during the menstrual cycle.

b. Materials and Methods

Testosterone and Androstenedione Superfusion Experiments

I have performed several experiments with proliferative and secretory endometrium, using the metabolically related compounds [1β-2β-^{3}H]-testosterone (T) (S.A. = 40 Ci/mmol) and [4-^{14}C]-androstenedione (Δ) (S.A. = 57.5 mCi/mmol). In four of the experiments, the concentration of these tracers in the superfusion medium was approximately 330,000 cpm/ml (11 pmol/ml) of ^{3}H-T and 30,000 cpm/ml (358 pmol/ml) of ^{14}C-Δ, with a ^{3}H/^{14}C ratio of 11. In the other experiments, tracer concentrations were slightly higher. The perfusion

ratios and tissue types for these experiments can be found listed in Table 6. The tissue specimens are dated as mid-proliferative (M.P.), late proliferative (L.P.) and secretory (S) with the day of the cycle noted. One tissue specimen (M21) could not be dated because it showed evidence of inflammation (endometritis).

The tissue specimens were prepared for superfusion in GBSS as previously described in the section on general materials and methods and were perfused for two hours at a flow rate of 15 ml/hr. Flow Chart 2 describes the procedure for the T and Δ superfusion experiments. Following the superfusion, the tissue fragments were rapidly washed 5 times with cold saline and homogenized in 10 ml of methanol containing carrier (250 or 500 μg) for testosterone, androstenedione, androstanedione (Adione), 5α dihydrotestosterone (DHT) and in the later experiments, androsterone. Aliquots (3 or 4 ml) of the superfusate, collected separately in 3 fractions during the second hour of perfusion, also received these carriers. The precipitate obtained following homogenization was separated by centrifugation at about 1200 x g for 10 minutes and the pellet was saved for protein determination. The supernatant was dried under nitrogen and the residue was partitioned between a water and ethyl acetate mixture; the aqueous layer was reextracted twice with 4 ml of ethyl acetate. A small aliquot of these extracts was counted for radioactivity and the rest was chromatographed on TLC silica gel plates using the solvent system TLC-1. The resultant bands of radioactive carrier were located by comparing the relative mobility of the extracted steroids to that of standards run in parallel and sprayed with 5% H_2SO_4 in ethanol. In addition, the

Table 6

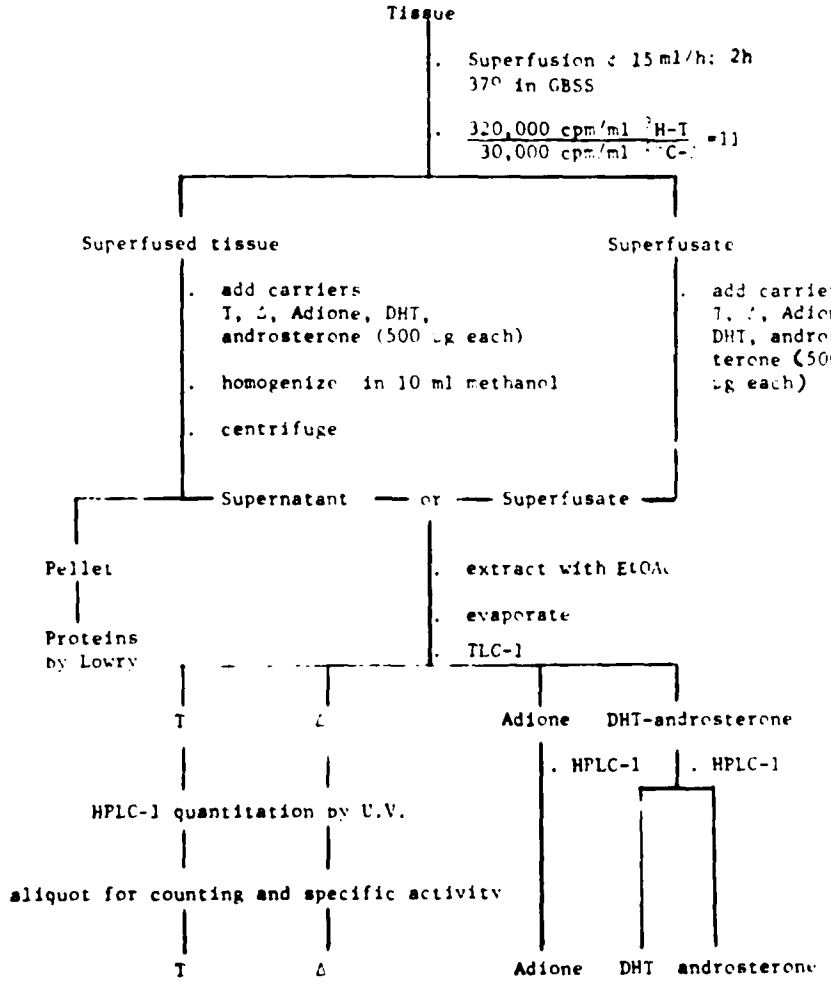
Experimental Results: Testosterone and Androstenedione Superfusion Experiments

Exp. #	Tissue	Concentration of tracers in perfused medium		Concentrations of tracers in superfusate		Concentration of tracers in tissue after superfusion			
		cpm/ml	pmol/ml	T / Δ ^3H cpm/ml / ^{14}C cpm/ml		T / Δ ^3H cpm/g / ^{14}C cpm/g		T pmol/g	Δ
F28	M.P.	^3H_T / $^{14}C_\Delta$ 312,000 / 29,000	=11	10.4 / 346		276,000 / 85	14,600 / 26, 00	311,000 / 1600	393,000 / 66,000	29.5	800
A26	M.P.	^3H_T / $^{14}C_\Delta$ 489,000 / 47,000	=10	16.3 / 559		431,000 / 100	21,200 / 46,000	604,000 / 2000	184,000 / 45,000	42.6	562
J24	L.P.	^3H_T / $^{14}C_\Delta$ 315,000 / 29,500	=11	10.4 / 353		272,000 / 150	23,000 / 29,000	239,000 / 6000	510,000 / 66,000	77.3	803
J10	S-22	^3H_T / $^{14}C_\Delta$ 314,000 / 28,400	=11	10.4 / 339		225,000 / 200	70,000 / 27,600	79,000 / 5000	667,00 / 65000	59.0	800
013	S-24	^3H_T / $^{14}C_\Delta$ 540,000 / 30,000	=18	18.0 / 356		285,000 / 430	197,000 / 27,800	106,000 / 4000	$2.5x10^6$ / 150,000	52.0	1872
M21	Endometritis	^3H_T / $^{14}C_\Delta$ 319,000 / 27,500	=12	10.8 / 328		257,000 / 130	39,300 / 25,700	70,000 / 600	382,000 / 42,000	9.4	520

M.P., mid-proliferative endometrium; L.P., late proliferative endometrium; S-number, secretory endometrium and day of menstrual cycle.

Flow Chart 2

Experimental Design; Testosterone and Androstenedione Superfusion Experiments



T and Δ were viewed due to their U.V. absorption. Dihydrotestosterone and androsterone are not separable by TLC-1 but were separated later by HPLC. The steroid bands were scraped from the TLC plates, eluted twice with 4 ml of ethyl acetate and an aliquot was counted.

The specific activity of the T and Δ in the tissue extract was determined after TLC and HPLC by radioactivity measurements combined with quantitation by U.V. spectrophotometry at 240 nm (Beckman DU-2), using Allen corrections (124). These data allowed the estimation of losses of radioactive compounds during extraction and purification. The other metabolites for which carriers were added to the tissue, viz. DHT, Adione and androsterone were purified by HPLC, and after further addition of carrier (2 to 5 mg) were crystallized. In five of the experiments, the same metabolites extracted from the superfusate were crystallized after addition of carriers and the $^{3}H/^{14}C$ ratios were compared to those in compounds isolated from tissue.

c. Results

The isotope data from several of the testosterone and androstenedione superfusion experiments can be found in Table 6 and crystallization data for these steroids isolated from the endometrial tissue are presented in Table 7. These data were used to calculate the parameters listed in Tables 8 and 9. These parameters include the fraction of the superfused tracer T or Δ which enters the tissue; the fraction of T which is converted in the tissue to Δ (or corresponding conversion of Δ to T), the fraction of the superfused tracer T or Δ, which having entered the tissue returns to the superfusate, and the

Table 7

Isotopic Data from Crystallization of Testosterone and Androstenedione Isolated from Tissue

Testosterone and Androstenedione Superfusion Experiments

Exp. #	Compound	Sample	Parameter			
			$^3H/^{14}C$	Sp. act. (cpm/mg) ^3H	^{14}C	Weight of crystals (mg)
F28	T	last chromatography	124			
		1st crystallization	184	8300	45	2.0
	Δ	last chromatography	5.7			
		1st mother liquor	6.2	5600	900	
		2nd mother liquor	6.4	5400	850	
		1st crystallization	5.8	5800	1000	0.9
		2nd crystallization	6.0	6000	1000	1.0
A26	T	last chromatography	356			
		1st crystallization	286	10,000	35	2.0
	Δ	last chromatography	3.8			
		1st crystallization	4.0	1700	425	1.0
		2nd crystallization	4.1	1300	320	0.9
		3rd crystallization	4.0	1600	400	1.1
J24	T	last chromatography	36			
		3rd mother liquor	46	4400	95	0.2
		1st crystallization	36	5000	140	1.1
		2nd crystallization	47	4200	90	1.0
		3rd crystallization	45	3600	80	1.1
	Δ	last chromatography	7.7			
		1st mother liquor	7.7	5800	750	0.1
		2nd mother liquor	7.7	5800	750	0.3
		1st crystallization	7.5	8300	1100	1.0
		2nd crystallization	8.0	8000	1000	0.7
		3rd crystallization	7.0	7000	1000	0.4
J10	T	last chromatography	14			
		1st mother liquor	14	1200	85	1.1
		1st crystallization	17	1000	60	0.9
		2nd crystallization	17	960	55	1.1
		3rd crystallization	16	475	30	1.2
	Δ	last chromatography	10.4			
		1st mother liquor	9.8	5700	580	0.7
		1st crystallization	10.0	7000	680	1.1
		2nd crystallization	10.0	7000	670	1.1
		3rd crystallization	10.0	6800	660	0.8
013	T	last chromatography	25			
		1st crystallization	30	600	20	0.8
		2nd crystallization	28	700	25	0.08

fraction of the perfused tracer T or Δ which returns to the superfusate as compound Δ and T, respectively. The equations to calculate these parameters can be found in the section on Calculations.

(i) Entry (α)

The concentration of tracers in the superfusion medium varies between 10 and 18 pmol/ml for testosterone and 328 and 559 pmol/ml for androstenedione (Table 6). In spite of this range in concentrations, the fraction of the superfused steroid taken up by the tissue, α_T and α_Δ, remains fairly constant (Table 8). Therefore, it appears that the steroid tracers, ^{3}H-testosterone and ^{14}C-androstenedione enter the cell by simple diffusion or using a transport carrier system with a high dissociation constant.

(ii) Exit (β/α, γ/α)

Having entered the cell, an average of 28% of the testosterone in the proliferative tissue and 3.5% in the secretory tissue is released $(\beta/\alpha)_T$ to the medium unmetabolized (Table 8). An average of 72% of the androstenedione is released, with no variation during the menstrual cycle. Therefore, more of the androstenedione than the testosterone is released by the cells into the medium unmetabolized. The testosterone, especially in the secretory endometrium, is rapidly converted to androstenedione in a partially reversible manner and leaves the tissue mainly as androstenedione.

This same preferential release of androstenedione is shown by the γ/α values for androstenedione and testosterone (Table 8). The γ/α is that percent of the superfused tracer entering the tissue in these experiments (either T or Δ) which appears in the superfusate as either

Table 8

Calculated Parameters: Testosterone and Androstenedione Superfusion Experiments

Exp. #	Endometrial specimen	α_T	β_T	α_Δ	β_Δ	$(\beta/\alpha)_T$	$(\beta/\alpha)_\Delta$	$\gamma_{T\Delta}/\alpha_T$	$\gamma_{\Delta T}/\alpha_\Delta$
F28	M.P.	0.17	0.05	0.17	0.09	0.30	0.53	0.29	0.018
A26	M.P.	0.19	0.07	0.14	0.11	0.40	0.80	0.21	0.014
J24	L.P.	0.16	0.02	0.18	0.09	0.13	0.50	0.44	0.028
J10	S-22	0.29	0.01	0.27	0.24	0.03	0.90	0.76	0.026
013	S-34	0.49	0.02	0.47	0.40	0.04	0.85	0.76	0.030
M21	endometritis	0.24	0.05	0.22	0.16	0.21	0.73	0.50	0.023

its oxidized or reduced metabolite (either Δ or T). In proliferative tissue, an average of 31% of the ^{3}H-T is released as ^{3}H-Δ while in secretory tissue, 76% is released as ^{3}H-Δ. The ^{14}C-Δ released as ^{14}C-T is very low throughout the cycle. This range of the values for the conversion and release of T as Δ in proliferative and secretory tissue emphasizes the metabolic differences between the two stages of the menstrual cycle.

(iii) Interconversion (ρ)

The intracellular conversion of T to Δ is extensive (Table 9) the oxidation of T to Δ ($\rho_{T\Delta}$) averages 43% in mid-proliferative tissue and 94% in secretory tissue. The reduction of Δ to T ($\rho_{\Delta T}$) averages 5 and 72%, respectively. The larger ρs for the conversion of T to Δ than for the conversion of Δ to T in proliferative tissue indicates that the preferred direction of metabolism is oxidative. When 17β hydroxysteroid dehydrogenase activity is increased in secretory tissue, the ρs for both T to Δ and Δ to T are increased but still the preferred direction is toward Δ.

Another way of demonstrating the increase in the oxidation of testosterone to androstenedione in secretory tissue is by a comparison of the ratios of ^{3}H-Δ/^{3}H-T in secretory versus proliferative tissue (Table 9). The average ratio in secretory tissue is more than 12 times greater than the average ratio in proliferative tissue. The ^{14}C-Δ/^{14}C-T ratios do not show a pattern which can be related to a phase of the menstrual cycle.

(iv) Uptake of Radioactivity by Tissue (T/M)

The end result of this increase in the oxidation of testosterone

Table 9

Calculated Parameters: Testosterone and Androstenedione Superfusion Experiments

Exp. #	Endometrial specimen	$\rho_{T\Delta}$	$\rho_{\Delta T}$	$^{3}H_{\Delta}/^{3}H_{T}$	$^{14}C_{\Delta}/^{14}C_{T}$	$(T/M)_{T}$	$(T/M)_{\Delta}$
F28	M.P.	0.55	0.06	1.26	41.0	0.99	2.27
A26	M.P.	0.30	0.04	0.30	22.5	1.20	1.00
J24	L.P.	0.77	0.22	2.10	11.0	0.76	2.20
J10	S-22	0.93	0.72	8.4	13.0	0.25	2.30
013	S-24	0.94	0.72	23.6	37.5	0.20	5.00
M21	endometritis	0.78	0.10	5.50	70.0	0.22	1.50

in secretory tissue is a decrease in the tissue concentration of testosterone. This decrease is evident when one compares the differences in distribution of ^{3}H-T between the tissue and medium $(T/M)_T$ during the cycle (Table 9). The parameter, T/M (uptake) in these experiments denotes the ratio of the concentration of the steroid in the tissue (cpm/g) to the concentration of that steroid in the superfusate (cpm/ml). The T/M for ^{3}H-T is lower in secretory tissue than in proliferative tissue.

The tissue concentrations of ^{14}C-Δ is unaffected by the increase in 17β hydroxysteroid dehydrogenase activity. This is shown by the relatively consistant tissue to medium distribution of ^{14}C-Δ $(T/M)_\Delta$ on Table 9. The similarity in $(T/M)_\Delta$ values is due to the fact that most of the androstenedione leaves the tissue; therefore, changes in the rate constant of conversion of T to Δ do not influence markedly the intracellular concentration of ^{14}C-Δ.

d. Quantitative Changes in the Metabolism of Testosterone and Androstenedione during the Menstrual Cycle

The rates of entry, interconversion and removal of T and Δ can be calculated for each of the experiments listed on Tables 8 and 9 by using equations 17-35. Since the rate values depend on the particular conditions under which each experiment was performed (e.g. weight of tissue superfused, concentrations of T and Δ in the superfusion medium, flow rate), results were normalized to arrive at meaningful conclusions. As described in the Introduction, rates can be calculated on the basis of conversion factors (ρ_{TA} and ρ_{AT}) and rates of entry of T and Δ into the tissue (V_{0T} and $V_{0\Delta}$). It is therefore possible to express the metabolic fate of T entering the tissue by assuming that $\rho_{T\Delta}$ and $\rho_{\Delta T}$ are constants for the tissue and that v_{0T} equals 100 and $v_{0\Delta}$ equals 0. In this case,

$$PR_T = 100$$

$$V_{T,T\bar{\rho}} = (\beta/\alpha)_T 100$$

$$V_{T,\Delta\bar{\rho}} = (\gamma_{T,\Delta\bar{\rho}}/\alpha_T)100$$

$$V_{T\Delta} = 100\rho_{T\Delta}/(1-\rho_{T\Delta}\rho_{\Delta T})$$

$$V_{\Delta T} = 100\rho_{T\Delta}\rho_{\Delta T}/(1-\rho_{T\Delta}\rho_{\Delta T})$$

$$V_{TM} = 100 + V_{\Delta T} - V_{T\Delta} - V_{T,T\bar{\rho}}$$

and

$$V_{\Delta M} = V_{T\Delta} - V_{\Delta T} - V_{\Delta,\Delta\bar{\rho}}$$

Similarly, when $v_{0\Delta}$ = 100 and v_{0T} = 0, the fate of Δ entering the tissue is described by the following parameters:

$$PR_{\Delta} = 100$$

$$V_{\Delta,\Delta\bar{P}} = (\beta/\alpha)_{\Delta}\, 100$$

$$V_{\Delta,T\bar{P}} = (\gamma_{\Delta T}/\alpha_{\Delta})\, 100$$

$$V_{\Delta T} = 100\, P_{\Delta T}/(1 - P_{T\Delta} P_{\Delta T})$$

$$V_{T\Delta} = 100\, P_{\Delta T} P_{T\Delta}/(1 - P_{T\Delta} P_{\Delta T})$$

$$V_{\Delta M} = 100 - V_{TM} - V_{\Delta T} - V_{\Delta,\Delta\bar{P}}$$

and

$$V_{TM} = V_{\Delta T} - V_{T\Delta} - V_{T,T\bar{P}}$$

Figure 4 shows rates calculated with the data from two experiments, one corresponding to proliferative endometrium (F28) and the other to secretory endometrium (J10). The calculated rates for the other T and Δ superfusion experiments can be found in Table 10. As can be seen from this figure in which metabolism of T and Δ in proliferative and secretory tissue are compared, in proliferative endometrium about one-half of the ^{3}H-T entering the cells is converted to Δ and the other half is removed as T or other T metabolites. In contrast, the bulk of the metabolism of T in secretory endometrium occurs by conversion to Δ and removal from the tissue as Δ. Metabolism of Δ in proliferative endometrium occurs mainly be diffusion of Δ out of the cells and by conversion of Δ to 5α reduced metabolites, with only minor reduction to T. The reversible conversion of Δ to T in secretory endometrium is markedly elevated but still the main route of removal of Δ from the tissue is diffusion out of the cells.

Figure 4.

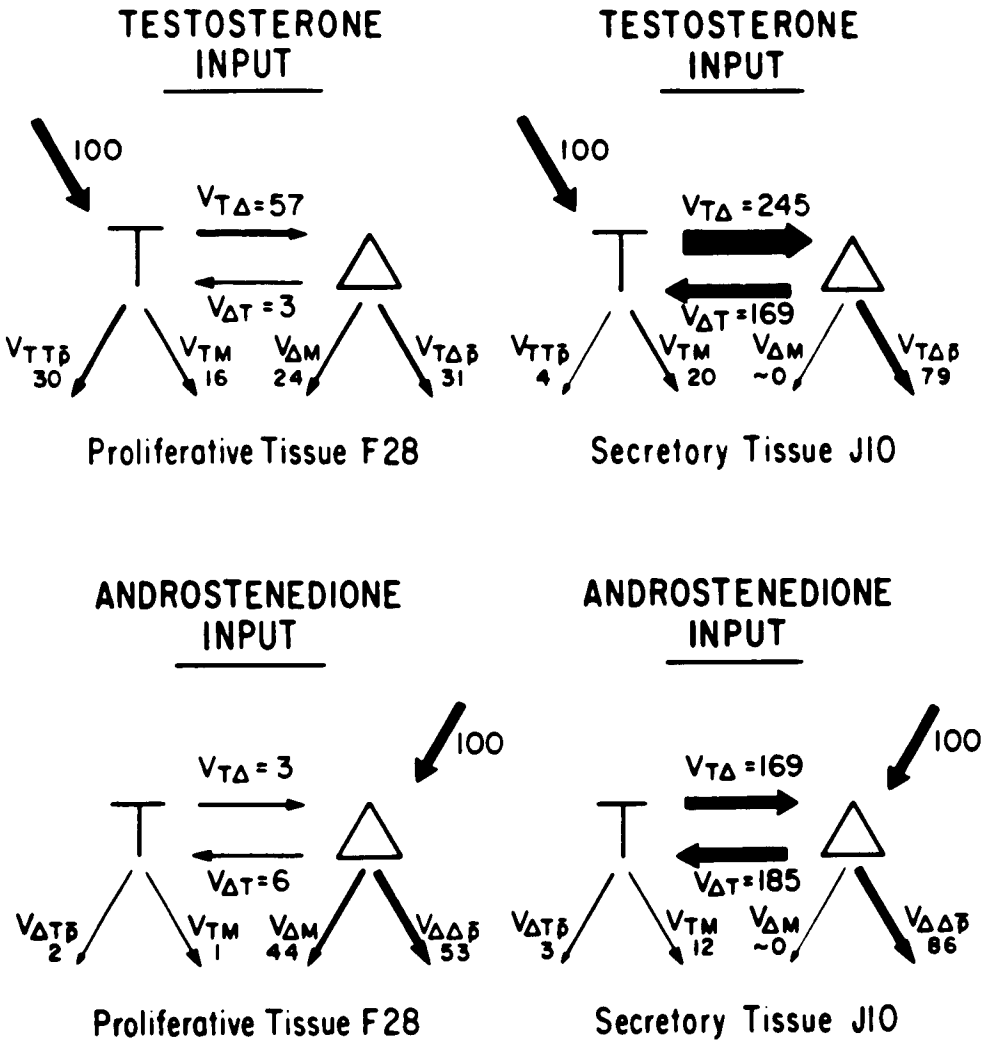

TESTOSTERONE INPUT
100
$V_{T\Delta}$=57
$V_{\Delta T}$ = 3
$V_{TT\beta}$ 30
V_{TM} 16
$V_{\Delta M}$ 24
$V_{T\Delta\beta}$ 31
Proliferative Tissue F28
TESTOSTERONE INPUT
100
$V_{T\Delta}$ = 245
$V_{\Delta T}$ =169
$V_{TT\beta}$ 4
V_{TM} 20
$V_{\Delta M}$ ~0
$V_{T\Delta\beta}$ 79
Secretory Tissue J10
ANDROSTENEDIONE INPUT
100
$V_{T\Delta}$ = 3
$V_{\Delta T}$=6
$V_{\Delta T\beta}$ 2
V_{TM} 1
$V_{\Delta M}$ 44
$V_{\Delta\Delta\beta}$ 53
Proliferative Tissue F28
ANDROSTENEDIONE INPUT
100
$V_{T\Delta}$ =169
$V_{\Delta T}$=185
$V_{\Delta T\beta}$ 3
V_{TM} 12
$V_{\Delta M}$ ~0
$V_{\Delta\Delta\beta}$ 86
Secretory Tissue J10

Table 10

Normalized Rates of Metabolism of T and Δ; Testosterone and Androstenedione

Superfusion Experments

Testosterone Input = 100

Exp. #	Endometr. specimen	$v_{T\Delta}$	$v_{\Delta T}$	$v_{TT_{\bar{p}}}$	$v_{T\Delta_{\bar{p}}}$	v_{TM}	$v_{\Delta M}$
F28	M.P.	56.9	3.4	30.0	29.4	16.5	24.1
A26	M.P.	30.4	1.2	40.0	21.0	30.8	8.2
J24	L.P.	93.0	20.4	13.0	44.0	14.4	28.6
J10	S-22	245	169	4.0	79.0	20.0	∿ 0
013	S-24	294	213	4.0	75.5	15.0	5.0

Androstenedione Input = 100

		$v_{T\Delta}$	$v_{\Delta T}$	$v_{\Delta\Delta_{\bar{p}}}$	$v_{\Delta T_{\bar{p}}}$	v_{TM}	$v_{\Delta M}$
F28	M.P.	3.4	6.2	53.0	1.8	1.0	44.2
A26	M.P.	1.2	4.0	80.0	1.4	1.4	17.2
J24	L.P.	20.4	26.5	50.0	2.8	3.3	44.0
J10	S-22	169	185	86.0	2.6	12.4	∿ 0
013	S-24	213	225	85.0	3.0	9.0	3.0

The changes in the metabolism of testosterone and androstenedione during the menstrual cycle can be related to changes in the 17β hydroxysteroid dehydrogenase activity. A comparison of the conversion rates of T to Δ and Δ to T between proliferative and secretory endometrial tissue in Fig. 4 points clearly to an increase in both rates in secretory tissue. In proliferative endometrium, the interconversion between T and Δ proceeds preferentially in the oxidative direction, and this preference is magnified in secretory tissue. The oxidation and removal of T as Δ is three times higher in secretory endometrium than in proliferative tissue. Concomitant with this is the low release of unmetabolized T by secretory tissue. The net effect of the increase in the rate constant of oxidation of T to Δ and its release as Δ in secretory tissue may result in a decrease in the tissue concentration of testosterone (see testosterone uptake values on Table 9).

Of interest in this analysis is experiment J24 which involves late proliferative tissue. It shows intermediate values between mid-proliferative and secretory tissue indicating that 17β hydroxysteroid dehydrogenase activity increases progressively as the tissue changes from proliferative to secretory. There are intermediate values for $\rho_{T\Delta}$ $\rho_{\Delta T}$, $(\beta/\alpha)_T$, $^3H\text{-}\Delta/^3H\text{-}T$, $(T/M)_T$ and $(\gamma T\Delta/\alpha T)$. All of these are affected by the level of 17β hydroxysteroid dehydrogenase activity which is increased by the progesterone secreted in large quantities by the luteal phase ovary.

Tseng and Gurpide (107) studied 17β hydroxysteroid dehydrogenase activity in both proliferative and secretory endometrium and found

that with estradiol as substrate, the former tissue formed 1.2 nmol of estrone per hr per mg of protein while the latter formed 14 nmol of estrone per hr per mg of protein. Norgestrel (17α ethynyl-18-methyl-19-nortestosterone), a progestin, was also effective in inducing enzymatic activity *in vitro* while *in vivo* induction of 17β hydroxysteroid dehydrogenase activity was observed in women in the follicular phase of the cycle who received oral progestin for several days (125).

The preferred substrate for 17β hydroxysteroid dehydrogenase in the endometrium is estrogen but it will use testosterone and androstenedione. The Michaelis-Menten constant for estradiol (E_2) is lower than for either testosterone or androstenedione and indicates a preference for the oxidation of estradiol to estrone (E_1) over the reverse reaction (123). This same study concludes that estradiol has the highest binding affinity for 17β hydroxysteroid dehydrogenase of all the steroids tested (E_1, E_2, T, Δ, progesterone, 20α-dihydroprogesterone, D and Δ^5 androstenediol).

In conclusion, the effect of the progesterone induced increase in 17β hydroxysteroid dehydrogenase activity in secretory tissue may result in a reduction of the endometrial concentration of testosterone by converting it to androstenedione which is mainly released without further metabolism. The physiological effect of this reduction in testosterone concentration can be considered anti-androgenic since androstenedione is a much less biologically active steroid than testosterone.

3. Formation of 5α Reduced C_{19} Metabolites

The 5α reduced metabolite of testosterone, dihydrotestosterone, must be considered a significant androgen in endometrial tissue since the reported human endometrial androgen receptor has a higher affinity for it than for testosterone (66,68,69). It was then considered of interest to study the endometrial accumulation and metabolism of the 5α reduced metabolites, dihydrotestosterone and androstanedione with the purpose of determining their relationship to their immediate precursors in endometrium, viz., testosterone and androstenedione, and to each other.

a. Experimental Design

A preliminary batch incubation experiment was performed with androstenedione in order to determine whether endometrial tissue is capable of producing DHT. Androstenedione was chosen as the substrate because its plasma concentration is 3 to 4-fold greater than that of testosterone and therefore it could serve as a major source of DHT in endometrial tissue. A batch incubation technique was chosen because for the concentration of radioactivity used, it produced a higher concentration of radioactive metabolites than the superfusion technique.

In the ^{3}H-testosterone and ^{14}C-androstenedione superfusion experiments already discussed, dihydrotestosterone, androstanedione and androsterone were isolated from the endometrial tissue and the superfusate for the purpose of determining the relative contribution of T and Δ to each of these reduced metabolites. It is possible with the isotopic data from these experiments to make an estimation of the fraction of T which is converted to a metabolite (DHT, for instance)

directly, and the fraction of T which is first oxidized to Δ before being converted to it. Similar estimations can be made of the fraction of Δ converted to DHT through T or via Adione. The calculations involved in making these estimations are described below.

A single superfusion experiment using ^{14}C-testosterone and ^{3}H-androstenedione was performed in order to reverse the 10 to 36-fold difference in endometrial tissue concentrations favoring Δ which was obtained during superfusions with the isotopes ^{3}H-T and ^{14}C-Δ. This concentration advantage of androstenedione probably influences the relative contributions of T and Δ to DHT. Therefore, the effect on DHT production of reversing the isotopes, and thus the relative tissue steroid concentrations, was studied.

Finally, in order to determine the conversion factors corresponding to the metabolism of T to DHT and of Δ to Adione, superfusion experiments were performed using combinations of ^{14}C-testosterone and ^{3}H-dihydrotestosterone or ^{14}C-androstenedione and ^{3}H-androstanedione.

b. Calculations of the Relative Quantitative Importance of Different Pathways of Conversion of T and Δ to 5α Reduced Metabolites

One can quantitatively evaluate the relative importance of the precursors testosterone and androstenedione to a metabolite (DHT) by knowing the $^{3}H/^{14}C$ ratios of the three steroids in tissue and the four intracellular specific activities of the two precursors (a^{3H}_{Tt}, a^{14C}_{Tt}, $a^{3H}_{\Delta t}$, $a^{14C}_{\Delta t}$). In most of these experiments the isotopes perfused were ^{3}H-T and ^{14}C-Δ. The following calculations can be used equally when the isotopes are reversed to ^{14}C-T and ^{3}H-Δ. The four specific activities in tissue are calculated from the isotopic data of an experiment as follows.

The specific activity of ^{3}H-T in tissue (a^{3H}_{Tt}) is the ratio of the concentration of ^{3}H in the tissue (cpm/g) to the total concentration of T in the tissue derived from both the ^{3}H-T perfused and the ^{14}C-Δ converted to T.

$$a^{3H}_{Tt} = \frac{C^{3H}_{Tt}}{\left(C^{3H}_{Tt} / a^{3H}_{T\,supf}\right) + \left(C^{14C}_{Tt} / a^{14C}_{\Delta\,supf}\right)} \qquad (36)$$

This equation is similar to equation 23 in the section on Calculations. The specific activity of ^{3}H-Δ in tissue ($a^{3H}_{\Delta t}$) is;

$$a^{3H}_{\Delta t} = \frac{C^{3H}_{\Delta t}}{\left(C^{3H}_{\Delta t} / a^{3H}_{T\,supf}\right) + \left(C^{14C}_{\Delta t} / a^{14C}_{\Delta\,supf}\right)} \qquad (37)$$

and since;

$$\frac{a_{Tt}^{3H}}{a_{Tt}^{14C}} = \left(3H/14C\right)_{Tt} \qquad (38)$$

the specific activities of ^{14}C-T and ^{14}C-Δ are:

$$a_{Tc}^{14C} = \frac{a_{Tt}^{3H}}{\left(3H/14C\right)_{Tt}} \qquad (39)$$

and

$$a_{\Delta c}^{14C} = \frac{a_{\Delta t}^{3H}}{\left(3H/14C\right)_{\Delta t}} \qquad (40)$$

Knowing these specific activities and the $^{3}H/^{14}C$ ratio in the metabolite (M), in this case DHT, it is possible to derive an equation for determining the ratio of the rate of conversion of T to the metabolite and Δ to the metabolite i.e. $v_{TM}/v_{\Delta M}$ in Fig. 5.

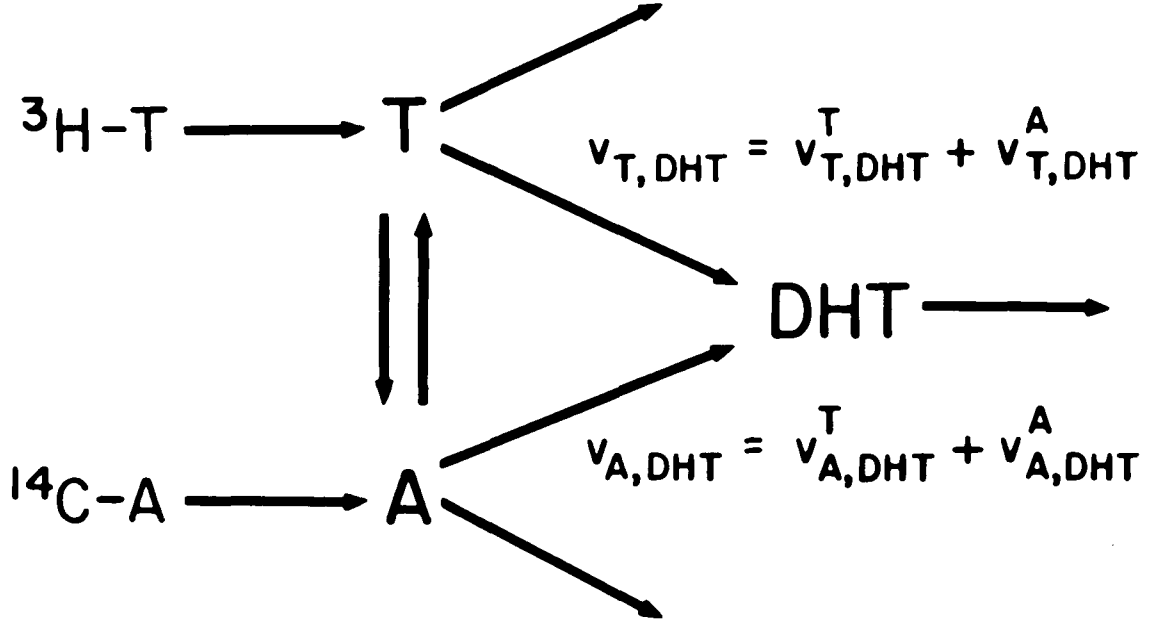
It should be noted in this scheme that the rate v_{TM} is made up of two components, the T that came originally from the ^{3}H-T perfused and the T from the ^{14}C-Δ perfused. Likewise, $v_{\Delta M}$ has two components. Thus,

$$V_{TM} = V_{TM}^{T} + V_{TM}^{\Delta} \qquad (41)$$

and

$$V_{\Delta M} = V_{\Delta M}^{T} + V_{\Delta M}^{\Delta} \qquad (42)$$

Figure 5.



where the superscripts indicate the original source of the T or Δ which is being converted to the metabolite.

At the isotopic steady state the $^{3}H/^{14}C$ ratio in the metabolite can be expressed as:

$$\left(^{3}H/_{^{14}C}\right)_{M} = \frac{V_{TM}\, a^{^{3}H}_{Tt} + V_{\Delta M}\, a^{^{3}H}_{\Delta t}}{V_{TM}\, a^{^{14}C}_{Tt} + V_{\Delta M}\, a^{^{14}C}_{\Delta t}} \qquad (43)$$

Dividing the numerator and denominator by $V_{\Delta M}$ and solving for $V_{TM}/V_{\Delta M}$, it follows that:

$$\frac{V_{TM}}{V_{\Delta M}} = \frac{\left(^{3}H/_{^{14}C}\right)_{M} a^{^{14}C}_{\Delta t} - a^{^{3}H}_{\Delta t}}{a^{^{3}H}_{Tt} - \left(^{3}H/_{^{14}C}\right)_{M} a^{^{14}C}_{Tt}} \qquad (44)$$

From the definitions of V^{T}_{TM} and V^{Δ}_{TM}, can say that:

$$V_{TM}\, a^{^{3}H}_{Tt} = V^{T}_{TM}\, a^{^{3}H}_{T\,supf} \qquad (45)$$

and

$$V_{TM}\, a^{^{14}C}_{Tt} = V^{\Delta}_{TM}\, a^{^{14}C}_{\Delta\,supf} \qquad (46)$$

Dividing equation 45 by equation 46, we arrive at:

$$\left(^{3}H/_{^{14}C}\right)_{Tt} = \frac{V^{T}_{TM}}{V^{\Delta}_{TM}} \quad \frac{a^{^{3}H}_{T\,supf}}{a^{^{14}C}_{\Delta\,supf}} \qquad (47)$$

or

$$\frac{V_{TM}^{T}}{V_{TM}^{\Delta}} = \left(\frac{^{3}H}{^{14}C}\right)_{Tt} \frac{a_{\Delta supf}^{^{14}C}}{a_{T supf}^{^{3}H}} \qquad (48)$$

Using the same reasoning and calculations in terms of the conversion of Δto M, we have:

$$\frac{V_{\Delta M}^{T}}{V_{\Delta M}^{\Delta}} = \left(\frac{^{3}H}{^{14}C}\right)_{\Delta t} \frac{a_{\Delta supf}^{^{14}C}}{a_{T supf}^{^{3}H}} \qquad (49)$$

Going back to our definitions of v_{TM}, equation 41 and $v_{\Delta M}$, equation 42, we can determine the percent of the Δ from superfused Δ which is converted to the metabolite via T as follows: From equation 41:

$$V_{TM} = \frac{V_{TM}^{T}}{V_{TM}^{\Delta}} V_{TM}^{\Delta} + V_{TM}^{\Delta} = \left(\frac{V_{TM}^{T}}{V_{TM}^{\Delta}} + 1\right) V_{TM}^{\Delta} \qquad (50)$$

and from equation 42:

$$V_{\Delta M} = \frac{V_{\Delta M}^{T}}{V_{\Delta M}^{\Delta}} V_{\Delta M}^{\Delta} + V_{\Delta M}^{\Delta} = \left(\frac{V_{\Delta M}^{T}}{V_{\Delta M}^{\Delta}} + 1\right) V_{\Delta M}^{\Delta} \qquad (51)$$

Dividing equation 50 by 51, we have:

$$\frac{V_{TM}}{V_{\Delta M}} = \frac{\left(\frac{V_{TM}^{T}}{V_{TM}^{\Delta}} + 1\right) V_{TM}^{\Delta}}{\left(\frac{V_{\Delta M}^{T}}{V_{\Delta M}^{\Delta}} + 1\right) V_{\Delta M}^{\Delta}} \qquad (52)$$

and rearranging equation 52, we arrive at:

$$\frac{V_{TM}^{\Delta}}{V_{\Delta M}^{\Delta}} = \frac{V_{TM}}{V_{\Delta M}} \frac{\frac{V_{\Delta M}^{T}}{V_{\Delta M}^{\Delta}} + 1}{\frac{V_{TM}^{T}}{V_{TM}^{\Delta}} + 1} \qquad (53)$$

in terms of the definitions in equation 47 and 48, it follows that:

$$\frac{V_{TM}^{\Delta}}{V_{\Delta M}^{\Delta}} = \frac{V_{TM}}{V_{\Delta M}} \frac{\left(^{3}H/^{14}C\right)_{\Delta t} \frac{a_{\Delta\,smpl}^{^{14}C}}{a_{T\,smpl}^{^{3}H}} + 1}{\left(^{3}H/^{14}C\right)_{Tt} \frac{a_{\Delta\,smpl}^{^{14}C}}{a_{T\,smpl}^{^{3}H}} + 1} \qquad (54)$$

In order to obtain an expression for the fraction (F) of Δ converted to M via T, i.e.

$$F_{V_{TM}^{\Delta}} = \frac{V_{TM}^{\Delta}}{V_{TM}^{\Delta} + V_{\Delta M}^{\Delta}} \qquad (55)$$

we divide the numerator and the denominator in equation 55 by $v_{\Delta M}^{\Delta}$.

$$F_{V_{TM}^{\Delta}} = \frac{\frac{V_{TM}^{\Delta}}{V_{\Delta M}^{\Delta}}}{\frac{V_{TM}^{\Delta}}{V_{\Delta M}^{\Delta}} + 1} \qquad (56)$$

and use the values for ratio $v_{TM}^{\Delta}/v_{\Delta M}^{\Delta}$ shown in equation 54.

Next, by dividing equation 48 by equation 49:

$$\frac{V_{TM}^{T}/V_{TM}^{\Delta}}{V_{\Delta M}^{T}/V_{\Delta M}^{\Delta}} = \frac{\left({}^{3}H/{}^{14}C\right)_{Tt}}{\left({}^{3}H/{}^{14}C\right)_{\Delta t}} \quad \frac{\frac{a_{\Delta sup f}^{{}^{14}C}}{a_{T sup f}^{{}^{3}H}} + 1}{\frac{a_{\Delta sup f}^{{}^{14}C}}{a_{T sup f}^{{}^{3}H}} + 1} \qquad (57)$$

and rearranging the equation, it is possible from the resultant expression to determine the relative importance of the pathways, v_{TM}^{T} and $_{\Delta M}^{T}$. The expression is:

$$\frac{V_{TM}^{T}}{V_{\Delta M}^{T}} = \frac{\left({}^{3}H/{}^{14}C\right)_{Tt}}{\left({}^{3}H/{}^{14}C\right)_{\Delta t}} \quad \frac{V_{TM}^{\Delta}}{V_{\Delta M}^{\Delta}} \qquad (58)$$

Using equation 54 to substitute, we have:

$$\frac{V_{TM}^{T}}{V_{\Delta M}^{T}} = \frac{\left({}^{3}H/{}^{14}C\right)_{Tt}}{\left({}^{3}H/{}^{14}C\right)_{\Delta t}} \frac{V_{TM}}{V_{\Delta M}} \frac{\left({}^{3}H/{}^{14}C\right)_{\Delta t}}{\left({}^{3}H/{}^{14}C\right)_{Tt}} \frac{a_{\Delta sup f}^{{}^{14}C}/a_{T sup f}^{{}^{3}H}}{a_{\Delta sup f}^{{}^{14}C}/a_{T sup f}^{{}^{3}H}} \qquad (59)$$

From this ratio, like equation 56, the fraction (F) of the superfused T converted to M via T can be determined as follows:

$$F_{V_{TM}^{T}} = \frac{V_{TM}^{T}}{V_{TM}^{T} + V_{\Delta M}^{T}} \qquad (60)$$

or

$$F_{V_{TM}^{T}} = \frac{\frac{V_{TM}^{T}}{V_{\Delta M}^{T}}}{\frac{V_{TM}^{T}}{V_{\Delta M}^{T}} + 1} \qquad (61)$$

In summary, knowing the specific activities of the two metabolically interrelated tracers perfused, the specific activities in the tissue of each of the four individually labeled precursors of the metabolite and the tissue $^{3}H/^{14}C$ ratio of both of the perfused compounds and their common metabolite, it is possible to determine the fraction of each of the precursors which is converted to the metabolite either directly or through its intermediate.

c. Materials and Methods

(i) Batch Incubation of Androstenedione

The batch incubation experiment consisted of an incubation of 140 mg of minced secretory endometrium for 3 hours with 131 pmol/ml (4.5×10^6 cpm/ml) of [1,2-^{3}H]-androstenedione (S.A. = 46.1 Ci/mmol) in 2 ml of GBSS. Following the incubation, carriers were added for Δ, T, DHT, Adione, androsterone and 5α androstane,3α,17β-diol (3α,17β-Adiol) (500 μg each) to both the tissue before homogenization and to the medium after its separation from the tissue. Extraction, purification, quantitation by R.I. or U.V. after HPLC and crystallization to a constant specific activity (after the addition of 5 mg of carrier) was carried out on the initial carrier steroids added to the tissue. The procedure followed that of the T and Δ superfusion experiments. The carriers added to the medium were also extracted, purified and quantitated after HPLC but not crystallized. A protein analysis was done on the homogenate pellet after steroid extraction.

(ii) Superfusions with Testosterone and Androstenedione

The ^{3}H-T and ^{14}C-Δ superfusion experimental procedure has been described previously, the details of the ^{14}C-T and ^{3}H-Δ superfusion experiment are as follows. Minced early proliferative tissue was superfused in GBSS containing 3.5 nmol/ml (0.26×10^6 cpm/ ml) of [4-^{14}C]-T (S.A. = 51.9 mCi/mmol) and 154.6 pmol/ml (5.3×10^6 cpm/ml) of [1,3-^{3}H]-Δ (S.A. = 46.1 Ci/mmol) for 1 1/2 hours at a flow rate of 8 ml/hour. Following the superfusion, carriers (500 μg) for T, Δ, DHT and Adione were added to both the tissue before homogenization and to the superfusate. The rest of the procedure for extraction, purifi-

cation and quantitation was the same as for the other T and Δ superfusion experiments except after HPLC the steroids were not crystallized. The homogenate pellet after extraction was analyzed for its protein content. Early proliferative endometrium was chosen for this experiment because reportedly (111) the highest levels of 5α reductase activity are found in endometrial tissue during the follicular phase of the menstrual cycle.

(iii) Superfusions with Dihydrotestosterone and Androstenedione

In one experiment, secretory endometrium (M-8) was superfused with a mixture of [4-^{14}C]-testosterone (S.A. = 51.9 mCi/mmol) at a concentration of 1.6 x 10^6 cpm/ml (21 nmol/ml) and [1,2-^{3}H]-5α-dihydrotestosterone (S.A. = 50.6 Ci/mmol) at a concentration of 725,000 cpm/ml (19 pmol/ml). In two other experiments, endometrial specimens were superfused with mixtures of labeled androstenedione and androstanedione. One of the specimens, early proliferative endometrium (F26)was superfused with [4-^{14}C]-androstenedione (S.A. = 55.75 mCi/mmol) at a concentration of 700,000 cpm/ml (8.7 nmol/ml) and [1,2-^{3}H]-5α-androstanedione (S.A. = 50.6 Ci/mmol/ml) at a concentration of 4 x 10^6 cpm/ml (106 pmol/ml). The other specimen, secretory endometrium (J11), was superfused with [4-^{14}C]-androstenedione (S.A. = 57.5 mCi/mmol at a concentration of 65,000 cpm/ml (775 pmol/ml) and [1,2-^{3}H]-5α-androstanedione at a concentration of 320,000 cpm/ml (8.5 pmol/ml). Each of these superfusions was carried out for two hours with a buffer flow rate of 8 ml/h.

The ^{3}H-androstanedione used for these experiments was prepared by oxidation of [1,2-^{3}H]-5α-dihydrotestosterone with an 0.2% solution

of chromic acid and acetic acid.

In experiments M8 and F26, carriers (500 μg) for T, Δ, DHT, Adione and androsterone were added to the tissues before homogenization and to the superfusates. In experiment F26, 5α-androstane 3α,17β-diol (500 μg) was added also as a carrier to the tissue and superfusate. In experiment J11, only DHT and Adione carriers (500 μg) were added to the pre-homogenized tissue.

The procedure for the extraction separation and crystallization of the steroids from both tissue homogenates and superfusates was the same as for the T and Δ experiments. Androgens were quantitated by HPLC, using the refractometer for measurement of the amount of carrier recovered.

d. Results

(i) Batch Incubations

The principal metabolite of androstenedione is testosterone. There is also considerable 5α reduction of androstenedione to androstanedione. The next most prominent metabolite is androsterone arising from the 3α reduction of androstanedione. Although the least abundant metabolite is dihydrotestosterone, comprising only 0.07% of the ^{3}H label incubated, the crystallization data (Table 11) offers proof that it is produced by endometrial tissue. The crystallization data for the carrier 5α-androstane-3α,17β-diol leads one to doubt that this steroid was produced by endometrial tissue in this particular experiment.

(ii) Pathways of Conversions of Testosterone and Androstenedione to Metabolites

Table 11

Isotopic Data from Crystallization of Steroids Isolated from Tissue

Batch Incubation ^3H_A

	Compound	C-1	C-2	C-3	ML-1	ML-2	ML-3
Specific activity ^3H (cpm/mg)	A	72,300	76,900	81,000	61,500	70,000	72,000
weight of crystals (mg)		1.0	1.0	1.8	0.3	0.2	0.0^4
Specific activity ^3H (cpm/mg)	T	3000	2600	2700	2700		2700
weight of crystals (mg)		1.0	1.1	1.0	0.2		0.2
Specific activity ^3H (cpm/mg)	DHT	400	250	250			240
weight of crystals ^3H (cpm/mg)		1.0	1.0	1.8			0.3
Specific activity ^3H (cpm/mg)	Adione	2300	2500	2500			2200
weight of crystals (mg)		1.0	1.1	1.3			0.07
Specific activity ^3H (cpm/mg)	Androsterone	1480	1470	1470	1750		
weight of crystals (mg)		1.1	1.1	1.6	0.4		
Specific activity ^3H (cpm/mg)	3α,17β-Adiol	88	49	32	231		366
weight of crystals (mg)		1.1	1.1	1.0	0.8		0.3

The $^{3}H/^{14}C$ ratios in the 5α reduced metabolites, DHT, Adione and androsterone isolated from these experiments are listed in Table 12. These ratios were usually determined after crystallization of the carriers to a constant specific activity but as noted in the table, there are some exceptions. The crystallization data for these compounds is in Table 13.

Referring to Table 12, it can be seen from the $^{3}H/^{14}C$ ratios that these three metabolites seem to be mostly derived from ^{14}C-Δ since their $^{3}H/^{14}C$ ratios are nearer to those of Δ than T. However, these ratios are not adequate evidence to make this conclusion.

It was of interest to estimate the fraction of the perfused ^{3}H-T which is directly 5α reduced to DHT as compared with the fraction which is oxidized to Δ and then 5α reduced and, conversely, the fraction of the perfused ^{14}C-Δ which is directly 5α reduced as compared to 17β reduced and then 5α reduced. The information needed to quantitatively evaluate the relative importance of these two pathways for each precursor in each experiment is the specific activity of the ^{3}H-T and ^{14}C-Δ perfused, the $^{3}H/^{14}C$ ratios in the tissue of DHT, T and Δ and the specific activities in the tissue of the ^{3}H-T, ^{14}C-T, ^{3}H-Δ and ^{14}C-Δ.

The results of these calculations for four of the ^{3}H-T and ^{14}C-Δ superfusion experiments can be found on Table 14. Only these four experiments have the complete data necessary to make the calculations which show that the preferred path of metabolism is through Δ to DHT. Over 90% of the intracellular ^{14}C-Δ is preferentially 5α reduced as compared to under 10% which is reduced first to T before ending as

Table 12

Androgen $^{3}H/^{14}C$ Ratios in Tissue: Testosterone and Androstenedione

Superfusion Experiments

Exp. #	Endometrial specimen	T	Δ	DHT	Adione	Androsterone
M26	E.P.	157*	4.0*	38*	4.6*	
F28	M.P.	184**	6.1	7.6*		7.4*
A26	M.P.	286**	4.0			
J24	L.P.	45	7.0	8.1	5.5	7.1
J9	L.P.	108	22	30*	17	11
J10	S-22	16	10	10.6	10	10
O13	S-24	28		2.7*		7.4*
M21	endometritis	127**	9.0	16**	8.9**	9.3**
J14	post pregnancy	61	4.4	24		

E.P., early proliferative; M.P., mid-proliferative; L.P., late proliferative; S-number, secretory endometrium and day of menstrual cycle.

* HPLC only.

** 1 crystallization. All other data correspond to constant $^{3}H/^{14}C$ in 2 or 3 consecutive crystallizations (Table 13).

Table 13

$^{3}H/^{14}C$ Ratios in Precursors and 5α-Reduced Metabolites Isolated from Tissue Superfused with ^{3}H-T and ^{14}CΔ: Crystallization Data

Exp. #	Compound	$^{3}H/^{14}C$ Ratios			
		Last chromatography	Crystallization C-1	C-2	C-3
J9	T	107	115	108	-
	Δ	23	23	22	-
	Adione	18	15	17	-
	Androsterone	28	16	11	-
J14	T	60	42	61	-
	Δ	4.0	4.0	4.4	-
	DHT	70	26	24	-
J24	DHT	4.3	9.3	10	8.1
	Adione	4.5	6.5	6.0	5.5
	Androsterone	5.2	8.0	7.0	7.1
J10	DHT	15	7.2	10	11
	Adione	8.4	10	10	-
	Androsterone	7.3	10	10	10

Table 14

Pathways of Conversion of Testosterone and Androstenedione to DHT

Exp. #	Endometrial specimen	Testosterone to DHT via T (%)	Testosterone to DHT via Δ (%)	Androstenedione to DHT via T (%)	Androstenedione to DHT via Δ (%)
F28	M.P.	20	80	1	99
J24	L.P.	8	92	1	99
J9	L.P.	35	65	10	90
J10	S-22	11	89	7	93

DHT. With intracellular ^{3}H-T, around 35% is directly 5α reduced, while over 65% is first oxidized to Δ before conversion to DHT.

The calculations, however, are very sensitive to a change in the $^{3}H/^{14}C$ ratio of the metabolite which is reflective in this case of the relative amounts of ^{3}H-T and ^{14}C-Δ which eventually end up as DHT.

What probably affects the relative conversion of each precursor to DHT in these experiments is the difference in concentration of T and Δ in the perfusion medium and thus in the tissue which takes up the steroids by simple diffusion. As can be seen from table 6, an approximately 33-fold difference in the medium concentration between T and Δ favoring Δ, results in an endometrial tissue concentration difference of between 10 to 36-fold still favoring Δ.

To determine whether, as expected, these concentration differences affected the relative amount of T and Δ which is converted to DHT, one superfusion experiment was carried out reversing the isotopes to ^{14}C-T and ^{3}H-Δ and thus created a tissue concentration difference favoring T by 4-fold. Table 15 contains the experimental results and calculated parameters of this experiment (F25).

These calculations indicate that 86% of the intracellular T was directly reduced to DHT as compared to an average of 18.5% in the ^{3}H-T and ^{14}C-Δ experiments. Yet in spite of this, 78% of the Δ was reduced to DHT probably through Adione. From these results, I concluded that the pathways of conversion of T or Δ to DHT in endometrial tissue are strongly affected by the intracellular concentrations of T and Δ when either has a greater tissue concentration, the more concentrated

Table 15

Experimental Results; ^{14}C-Testosterone and ^{3}H-Androstenedione Superfusion Experiments (F25)

Early Proliferative Endometrium

Concentration of tracers in perfusion medium		Concentration of tracers in superfusate		Concentration of tracers in tissue after perfusion			
		T	Δ	T		Δ	
cpm/ml	pmol/ml	^{3}H cpm/ml / ^{14}C cpm/ml	^{3}H cpm/ml / ^{14}C cpm/ml	^{3}H cpm/g / ^{14}C cpm/g	pmol/g	^{3}H cpm/g / ^{14}C cpm/g	pmol/g
$\frac{^{3}H_{\Delta}}{^{14}C_{T}}$ $\frac{5.3 \times 10^{6}}{259,000}$ =20	155 / 3453	97,000 / 205,000	3.9 x 10^{6} / 564,000	2.6 x 10^{6} / 841,000	11.3	9.2 x 10^{6} / 195,000	2.9

Calculated Parameters

Steroid	α	β	β/α	γ/α	ρ	T/M	Metabolite $^{3}H/^{14}C$ in tissue DHT	Metabolite $^{3}H/^{14}C$ in tissue Adione	Conversion T to DHT via T (%)	Conversion T to DHT via Δ (%)	Conversion Δ to DHT via T (%)	Conversion Δ to DHT via Δ (%)
Androstenedione	0.37	0.11	0.29	0.05	$\rho_{\Delta T}$0.15	1.7	20	42	0.86	0.14	0.22	0.78
Testosterone	0.33	0.12	0.37	0.14	$\rho_{T\Delta}$0.43	3.2						

substrate is reduced in larger amounts. Yet, even when there is a four-fold tissue concentration difference favoring testosterone, a majority of the androstenedione in the tissue is 5α reduced. This extent of 5α reduction of Δ might be the result of perfusing early proliferative endometrium where the rate of conversion of Δ to T is low, thus leaving most of the intracellular pool of Δ for 5α reduction to Adione.

The other parameters that can be calculated from the isotope data of superfusion experiment (F25) support the pattern of 17β hydroxy-steroid metabolism which I have already discussed. The tissue released T as T (37%) or as Δ (14%) and converted T to Δ ($\rho_{T\Delta}$ = 43%) just as would be expected from proliferative endometrium (Tables 8 and 9). Similarily, Δ is released as Δ (28%) or as T (5%) and is connected to T ($\rho_{\Delta T}$ = 15%) also reflective of proliferative endometrium.

(iii) Extent of 5α reduction of testosterone and androstenedione

The isotopic data from experiments in which 5α reduction of Δ and T (F26) and (M8) was measured can be found in Table 16. Data from the crystallization of the steroids isolated from tne tissue and superfusate of these two experiments plus a third experiment with ^{14}C-Δ and ^{3}H-Adione (J11) can be found in Table 17. From these data, I have calculated the transfer factors of the perfused T and Δ to their immediate 5α reduced metabolites. The ρ for T to DHT in secretory endometrium (M8) is 0.8% and the ρ for Δ to Adione in secretory tissue (J11) is 2.7%. I can not say whether these ρs are representative of proliferative endometrial tissue as well.

Experiment F26 whose isotopic data appears in Table 16 did not yield sufficient ^{14}C in the crystallized Adione (Table 17) to determine

Table 16

Experimental Results: Androstenedione and Androstanedione Superfusion Experiments

and Testosterone and Dihydrotestosterone Superfusion Experiments

Endometrial specimen (Exp. #)	Concentration of tracers in perfused medium cpm/ml (pmol/g)	Concentration of androgens in tissue cpm/g (pmol/g)					Concentration of androgens in superfusate cpm/ml (pmol/ml)				
		T	Δ	DHT	Adione	Andros.	T	Δ	DHT	Adione	Andros.
Early proliferative (F26)	^{3}H-Adione 4 x 10^{6} (112)			279,000 (7.4)	9.8×10^{6} (264)	1.6×10^{6} (44)			160,000 (0.5)	4.2×10^{6} (112)	35,000 (0.9)
	$^{14}C_{\Delta}$ 700,000 (8700)	13,500 (168)	513,000 (6380)		6500 (77)	1200 (14)	2300 (27)	637,000 (7580)	800 (9.5)	2000 (24)	60 (0.7)
Secretory Day 24 (M8)	$^{3}H_{DHT}$ 725,000 (19)			1.4×10^{6} (37)	1.6×10^{6} (42)	747,000 (19)			674,000 (18)	85,000 (2.3)	13,000 (0.3)
	$^{14}C_{T}$ 1.6×10^{6} (21,300)	712,000 (9500)	1.6×10^{6} (14,700)	24,500 (330)	71,000 (950)	20,000 (270)	1.4×10^{6} (18,700)	296,000 (4000)	1700 (23)	3000 (40)	300 (4)

Table 17

Isotopic Data from Compounds Isolated from Tissue Superfused with ^{14}C-Δ and ^{3}H-Adione or ^{14}C-T and ^{3}H-DHT

Exp. #	Compound	$^{3}H/^{14}C$ ratio after last chromatography	Crystallization Data: $\frac{\text{specific act. } ^{3}H}{\text{Specific act. } ^{14}C} = \frac{^{3}H}{^{14}C}$ ratio (mg crystals)		
			C-1	C-2	C-3
M8	T	∿ 0	0/11,000 ∿ 0 (1.0)	0/11,000 ∿ 0 (0.9)	0/10,000 ∿ 0 (1.8)
	Δ	∿ 0	0/20,000 ∿ 0 (1.0)	0/22,000 ∿ 0 (1.0)	0/20,000 ∿ 0 (1.5)
	DHT	42	21,000/375 = 56 (1.0)	20,000/375 = 55 (1.0)	21,000/375 = 55 (2.3)
	Adione	23	10,200/465 = 22 (1.0)	10,300/465 = 22 (1.0)	10,200/465 = 22 (1.6)
	Androsterone	33	14,400/400 = 38 (1.0)	14,400/400 = 36 (1.0)	13,600/360 = 38 (1.0)
F26	T	1.5	40/150 = 0.3 (1.0)	0/120 ∿ 0 (1.1)	0/130 ∿ 0 (1.3)
	Δ	0.03	0/5600 ∿ 0 (0.9)	80/5300 = 0.02 (1.0)	60/5400 = 0.01 (2.6)
	DHT	84	2900/6 = 480 (0.9)	2200/7 = 330 (0.9)	2200/0 ∿ ∞ (0.7)
	Adione	∞	110,000/10 = 1100 (1.0)	124,000/80 = 1550 (1.2)	113,000/75 = 1500 (1.7)
	Androsterone	∞	12,000/0 ∿ ∞ (1.0)	11,000/0 ∿ ∞ (0.9)	11,000/0 ∿ ∞ (1.3)
	3α,17β-Adiol	22.5	84/1 = 84 (1.0)	89/6 = 15 (1.0)	36/0 ∿ ∞ (0.7)
J11	DHT	7.0	400/50 = 8.0 (1.0)	200/25 = 8 (1.1)	200/25 = 8 (1.5)
	Adione	7.5	5000/600 = 8.3 (1.0)		5000/600 = 8.3 (1.1)

accurately the conversion factor ρ_{Δ}-Adione, which can only be estimated to be less than 0.5%. Therefore,the experiment was repeated (J11) with a lower $^{3}H/^{14}C$ perfusion ratio (0.2) so that there would be sufficient ^{14}C in Adione to calculate the ρ_{Δ} Adione.

Experiment F26 yielded my first positive evidence for the production of 5α-androstane-3α,17β-diol by endometrial tissue. Table 18 shows the data for the crystallization of the carrier 3α,17β-Adiol added to the superfusate of the last two 20 minute periods of the superfusion. The specific activities of the 3α,17β-Adiol crystals from the superfusate are low but consistent.

The similarity of the specific activity of the mother liquor and the crystals for the final crystallization of the last aliquot indicates that this compound is radiochemically pure. The recovery of the 3α,17β-Adiol carrier after HPLC in these two aliquots was 67 and 21%. When the correction is made for this recovery after crystallization with 5 mg of additional carrier, there is probably 1300 cpm of ^{3}H-3α,17β-Adiol in this superfusate.

(iv) Distribution of Radioactivity in Tissue among 5α Reduced Metabolites

The relative proportions of radioactive metabolites in tissue derived from each of the labeled precursors used in the superfusions F26 and M8, T, Δ, DHT and Adione are shown in Table 19. This table provides us with an idea of the comparative concentrations of metabolites which could be found in endometrial tissue after exposure to these four steroids. We can see that in early proliferative tissue (F26), there is a relatively small amount of T from Δ. This supports

Table 18

Isotopic Data from Crystallization of 3α,17β-Androstanediol Isolated from Superfusate Androstenedione and Androstanedione Superfusion Experiment

Superfusate #	Sample	Parameter			
		$^{3}H/^{14}C$	Specific act. (cpm/mg) ^{3}H	^{14}C	Weight of crystals (mg)
Penultimate	last chromatography	8.8			
	1st mother liquor	9	28	9	1.2
	1st crystallization	∞	53	0	0.7
	2nd crystallization	∞	49	0	0.5
	3rd crystallization	∞	41	0	1.2
Ultimate	last chromatography	9.2			
	1st mother liquor	7.5	112	15	2.5
	2nd mother liquor	6.4	32	5	1.9
	1st crystallization	∞	56	0	0.7
	2nd crystallization	∞	41	0	0.5

Table 19

Distribution of Radioactivity Among Intracellular Metabolites of the Superfused Labeled Precursor

Exp. #	Endometrium	Precursor	Intracellular Compound				
			T	Δ	DHT	Adione	Androsterone
F26	early proliferative	Δ	2.6	100	∿ 0	1.3	0.2
		Adione	-	-	2.8	100	16
M8	secretory (d 24)	T	100	154	3.4	10	2.8
		DHT	-	-	100	114	53

the earlier discussed evidence for lower levels of 17β hydroxysteroid activity in proliferative tissue. In the secretory tissue (M8), as would be expected, there is considerable metabolism of T to Δ and possibly because of this larger pool of Δ (15 nmol/g) than T (9.5 nmol/g) there is three times more of its 5α reduced metabolite Adione in the tissue than DHT. This is consistent with the pattern of production of DHT through Δ to Adione found in the series of T and Δ superfusion experiments, when the tissue concentration of Δ was much larger than the tissue concentration of T. Also from this distribution of radioactivity, it is evident that in both proliferative and secretory endometrial tissue, both DHT and Adione are further converted to androsterone.

e. Discussion

Testosterone and androstenedione are both 5α reduced in proliferative and secretory tissue. This 5α reduction is important because through it DHT which is the physiologically active androgen in many target tissues can be produced both directly from testosterone and indirectly from androstenedione. Calculations from these data indicate that the relative importance of these two pathways varies depending upon the intracellular concentration of the two precursors, T and Δ. When the intracellular concentration of Δ is several fold greater than the concentration of T, the preferred pathway for 5α reduction seems to be via Δ. Then the intracellular concentration differences between T and Δ are reversed, the larger concentration of T relative to Δ results in the predominance of the pathway of direct conversion of T to DHT. However, even under these circumstances, most of the intra-

cellular Δ which is converted to DHT is converted through Adione.

Once 5α reduced, T and Δ are further converted to androsterone and 3α,17β-Adiol. Thus, from the results of these experiments it is evident that enzymes exist in endometrial tissue which are able to fully reduce androgens.

In summary, T and Δ are 5α reduced to DHT and Adione, respectively, with conversion factors of 0.8 and 2.7%. The DHT and Adione are further metabolized by either 17β hydroxysteroid dehydrogenase or 3α hydroxysteroid dehydrogenase. The 17β hydroxysteroid dehydrogenase interconverts DHT and Adione with a preference toward oxidation as suggested by a comparison of the ratios of concentrations of labeled DHT and Adione obtained when either DHT or Adione were superfused (Table 19). The 3α hydroxysteroid dehydrogenase reduces the Adione to androsterone probably before much of it can be 17β reduced to DHT and some 3α,17β-Adiol is produced from the androsterone. The net result of this metabolism in endometrial tissue is a small amount of DHT which may be biologically active.

III. GENERAL CONCLUSIONS ON ENDOMETRIAL ANDROGEN METABOLISM

In this series of experiments, the presence in endometrial tissue of 5 enzymatic activities metabolizing androgens has been demonstrated. A steroid alcohol sulfatase hydrolyzes dehydroepiandrosterone sulfate to dehydroepiandrosterone, a Δ^5,3β-ol isomerase-dehydrogenase complex converts dehydroepiandrosterone to androstenedione, a 17β hydroxysteroid dehydrogenase reversibly reduces androstenedione to testosterone and androstanedione to dihydrotestosterone, a 5α reductase reduces androstenedione and testosterone to Adione and DHT and, finally, a 3α hydroxysteroid dehydrogenase reduces androstanedione to androsterone and likely dihydrotesterone to 5α-androstane-3α,17β-diol (Fig. 1). W.P. Collins et al first reported evidence for the existence of these enzymes in human endometrial tissues in 1968 (126).

The activities of some of these enzymes have been found to vary during the course of the menstrual cycle and therefore they are presumed to be controlled by ovarian steroids. In the case of 17β hydroxysteroid dehydrogenase, progesterone has been found by Tseng and Gurpide (107, 109) and Pollow et al (108) to increase its activity during the secretory phase of the menstrual cycle. This increase in 17β hydroxysteroid dehydrogenase activity in secretory tissue is indirectly confirmed by the data showing an increase in the rate constants of both the oxidation of testosterone to androstenedione and the reduction of androstenedione to testosterone with the preferred direction of metabolism toward oxidation.

As a consequence of this increase in oxidation of testosterone in endometrial tissue after ovulation there is a decrease in the tissue

to medium concentration ratio of testosterone $(T/M)_T$. The tissue to medium concentration ratio of androstenedione $(T/M)_\Delta$ varies little during the cycle, possibly because the removal of androstenedione through metabolism to testosterone in proliferative and secretory tissue is relatively minor in comparison to the removal of androstenedione from the tissue by diffusion.

It was found that in proliferative tissue, approximately half of the testosterone entering the cells is converted to androstenedione and half leaves the tissue either as testosterone or other metabolites. In secretory tissue most of the testosterone is converted to androstenedione and leaves the tissue as androstenedione. In proliferative tissue, the androstenedione which has entered the cells is removed unmetabolized or is 5α reduced to androstanedione. Only a small amount is irreversibly reduced to testosterone. The pattern is the same in secretory tissue, with greater reversible conversion of androstenedione to testosterone.

Whether there is a change in 5α reductase activity in the endometrium during the menstrual cycle is still uncertain. According to several studies (110,127,128) there is no variation in activity. However, Pollow et al (111) found the highest levels of 5α reductase activity in early proliferative endometrium. The data reported here demonstrates that DHT is produced by both secretory and proliferative tissue directly from testosterone and indirectly from androstenedione. The relative amount of DHT derived from either testosterone or androstenedione depends largely upon the relative intracellular concentrations of the precursor compounds. When the intracellular

concentration of androstenedione exceeds that of testosterone, the conversion of testosterone to DHT occurs preferentially by an indirect pathway including androstenedione and androstanedione as intermediates. The direct pathway becomes important as the concentration of testosterone relative to androstenedione increases. The predominance of one pathway of testosterone to DHT over the other may reflect differences in the K_m of androstenedione and testosterone for the endometrial 5α reductase and differences in the pool sizes of these two precursors. However, even when the preferred pathway is the direct reduction of testosterone to DHT, most of the androstenedione metabolized by proliferative tissue is converted to DHT. The biological significance of these two pathways to DHT can not be evaluated at this moment since the relative endogenous concentrations of testosterone and androstenedione in endometrial tissue are unknown.

The metabolism of androgens by endometrial tissue contributes to the formation of a small intracellular pool of physiologically active DHT from circulating plasma precursors. The results obtained indicate that 10 to 20% of the dehydroepiandrosterone sulfate reaching the cells is hydrolyzed to dehydroepiandrosterone and that 3% of the intracellular dehydroepiandrosterone is converted to androstenedione (129). This androstenedione from dehydroepiandrosterone and the androstenedione and testosterone taken up by the endometrial tissue from plasma are 5α reduced, Δ to Adione (2.7%) and T to DHT (0.8%).

The pool of DHT may become smaller in secretory tissue because of the competition of testosterone and androstenedione with progesterone for 5α reductase (130). Kinetic studies of testosterone 5α

reduction and its competitive inhibition indicate a slightly greater affinity of 5α reductase for progesterone than for testosterone (131). The competition of progesterone for 5α reductase along with its stimulation of 17β hydroxysteroid dehydrogenase activity could have an adverse effect upon the levels of DHT in endometrial tissue as it does upon the levels of estradiol. Consequently, 17β hydroxysteroid dehydrogenase might be considered to have an antiandrogenic effect as well as an antiestrogenic effect on endometrial tissue.

IV. ANDROGENS AND ENDOMETRIAL ADENOCARCINOMA

(a) Introduction

In the rat, the primary research animal for studying androgen-uterine interaction, the most recently reported findings suggest

> "that the immature rat uterus is responsive to androgen stimulation by interactions with both the androgen and estrogen receptor systems, with a critical dependence on androgen dose and injection vehicle. Lower doses of T or DHT (200-500 μg) appear to induce modest growth via involvement of the androgen-receptor system predominantly. When higher doses of androgens are administered in a slow-release oil vehicle, estrogen-receptor movement and nuclear interaction of this receptor becomes apparent, suggesting that this receptor system may then mediate further the androgen-evoked uterotrophic response" (81). One can not extrapolate these conclusions on the rat to the human.

Whether normal concentrations of circulating androgens have a physiological effect on the human uterus is not known. Exogenous testosterone will either not alter normal uterine histology and cyclic menstruation or will cause uterine atrophy depending upon the dose administered. Five hundred milligrams or more of testosterone propionate will reduce the endometrium to a state of inactivity (132). The histological picture as described by Huffman (133)was of "thinning of the endometrium with very few glands in a non-secretory state. These glands were lined by a single layer of cells with small basal nuclei." Doses of this magnitude (usually given as 25 to 50 mg, 3 times per week for a month) (134) also induced masculinization (135). A smaller dose of 300 to 400 mg per month preserved the appearance

of a proliferative endometrium but usually delayed menstrual bleeding for a few days and caused a scant flow (135). The minimal dose given (30 to 120 mg per month) apparently did not disturb menstrual rhythm and the endometrium showed a characteristic secretory pattern (136). Androgens were given to women to treat many gynecologic conditions including abnormal uterine bleeding, functional dysmenorrhea and menopausal complaints.

The use of testosterone to treat these gynecologic problems ceased decades ago and is looked back on now by clinicians with embarrassment. Its previous use though gives us an opportunity to compare the effects of exogenous androgens upon humans and research animals. The largest doses of testosterone given to women were smaller comparatively than the hyperstimulatory doses given to rodents. Therefore, the atropic effects upon the human uterus may correspond to the low dose inhibitory effects of androgens seen in rodents.

If increasing circulating androgen concentrations by exogenous testosterone can cause uterine atrophy, the effects of long term exposure of the human uterus to elevated endogenous androgen concentrations can only be surmised by observing the uterus in cases of chronic androgen hyperproduction.

A major source of androgens in women is the ovary. In the normal human ovary (studied in vitro) all cell types, granulosa, theca and stroma have the synthetic capacity to produce progesterone, androgens and estrogens. The pattern of production of these steroids varies depending upon the stage of maturation or atresia of each

individual follicle (137). In fact, except for the large antral follicles (> 10 mm diameter), these cell types preferentially metabolize Δ to DHT instead of to estrogen (138). In vivo, the theca interna under LH control favors androgen production (139) as do granulosa cells in degenerating follicles, where they lose their capacity to produce estrogens and progesterone (140). Thus, the normal human ovary is a major source of circulating androgens since most of the thecal secretions enter the blood stream (137).

The most common source in women of chronic androgen hyperproduction is the abnormal ovary. The uterine response to this hyperproduction may be manifested by an increased incidence of endometrial carcinoma in association with ovarian pathology. The evidence for this association has been noted and documented since 1922 (141,142). It has been concluded that thecal cell tumors are nine times more commonly found in association with endometrial carcinoma than are found in women without any endometrial pathology (143).

Stromal cell hyperplasia also frequently accompanies endometrial carcinoma (144,145,146,147,148). The incidence of stromal hyperplasia with endometrial cancer in these studies ranges from 52% of 44 postmenopausal women (145) to 87% of 307 postmenopausal women (147). Several reports of the inverse of this relationship also have been published; that is a high incidence of endometrial hyperplasia and/or carcinoma with ovarian tumors (149,150,151,152). Arthur Hertig concluded from his vantage point of over 50 years of medical experience that "the probability (24%) that a patient older than 50 who has a granulosa cell-theca cell tumor will develop endometrial carcinoma

is 17 times higher than for the patient without such a tumor" (153).

Endometrial carcinoma is primarily a disease of postmenopausal women and the preceding discussion pertains principally to them. Only 4% of the cases of endometrial carcinoma occur in women under 40 years of age (154). Among this 4% are young women who have polycystic ovarian disease, or what was initially described by Drs. Stein and Leventhal (155,156) and named after them, the Stein-Leventhal syndrome. This syndrome is characterized by secondary amenorrhea, sterility, bilateral fluid-filled polycystic ovaries and a male type of hirsutism occurring during the second and third decade of life. All women who have this rare syndrome have polycystic ovaries but not all young women with polycystic ovaries have the syndrome.

A survey of medical literature has uncovered 51 reported cases of Stein-Leventhal syndrome with adenocarcinoma (157). Several reports have been made also of the combination of polycystic ovarian disease and endometrial carcinoma (154, 158) or endometrial hyperplasia (159,160).

The special feature of polycystic ovarian disease (and Stein-Leventhal syndrome) of interest here is the hyperandrogenism which accompanies it. Elevated plasma levels of unbound T, and total Δ and T have been reported (161). The source of this T and Δ as determined by Kirschner and Jacobs (162), who performed bilateral adrenal and ovarian catheterization on 13 hirsute women was direct ovarian secretion of T, as well as ovarian and adrenal Δ that was converted to T peripherally.

The etiology of polycystic ovarian disease has been explained

by Yen et al (163) as a probable disturbance in hypothalamic regulation consisting of high base line serum LH levels with a pattern of erratic fluctuations, while FSH concentrations are low. The end result is chronic anovulation and abnormal ovarian steroidogenesis.

Aside from the ovaries, the other potential source of androgen hyperproduction in young women is the adrenal gland. Atypical endometrial hyperplasia has been reported in association with an adrenocortical tumor and increased testosterone production (164). In a group of 37 women who died of endometrial carcinoma, eighty-four percent was found to have adrenal cortical hyperplasia upon autopsy (148).

In addition to pathological states, self-induced increases in circulating androgens have been associated with endometrial carcinoma. There have been recent reports of the development of endometrial adenocarcinoma after the 4 to 8 year use of the sequential oral contraceptive, Oracon (165,166). This was the only oral contraceptive to contain dimethisterone as the progestational agent (166) and is the only contraceptive to be directly associated with endometrial carcinoma. Dimethisterone (17β-hydroxy-6α-methyl-17-(1-propynyl)-andros-4-ene-3-one) carries a 17β hydroxyl group which is responsible for its androgenic activity.

The concern of the foregoing discussion has been a description of some pathology causing elevated androgen production and its association with endometrial carcinoma. These disease states are acknowledged risk factors for endometrial cancer (167). One remaining risk factor to consider is obesity. Obesity is the most common

clinical correlate of endometrial cancer with a reported incidence of 21 to over 80% (168,169,170). Ernest Wynder et al concluded that a women who is 50 lbs overweight has a nine times greater risk of developing endometrial cancer than a woman whose weight is normal (171).

How might obesity be related to hyperandrogen production and endometrial cancer? The association between obesity and endometrial carcinoma is commonly accepted as an association between obesity, fat conversion of Δ to E_1 and endometrial carcinoma. There is a hypothesis that obese postmenopausal women whose ovaries no longer produce estrogens (172,173,174), but continue to produce T (as premenopausally) and Δ (decreasingly) (175,176), and young women with erratic menstrual histories, anovulatory cycles, polycystic ovarian disease and elevated androgen production have in common a preponderance of estrone production, via extraglandular aromatization of Δ. This estrone chronically stimulates the endometrium of these women without the relief of periodic sloughing and may therefore promote endometrial cancer. The rationale for this "estrone hypothesis" to quote MacDonald and Siiteri (177) is "that those conditions that favor the possibility for increased estrone production at extraglandular site(s) are the same as those conditions in which an increased occurrence of endometrial carcinoma has been observed", i.e. aging and obesity.

A relationship between aging and obesity was initially supported by the conclusion that fat, a major site of peripheral aromatization of androgens (178,179), with Δ as the immediate precursor (180) and

estrone as the near exclusive product (181) (though T and DHT also have been isolated from adipose tissue) (182) will, with aging, progressively and significantly increase its efficiency of aromatization (183,184). In young non-obese women between 0.7 and 1.3% of infused Δ is converted to E_1 (185,186), while non-obese postmenopausal women convert about 2.7% of the Δ to E_1 (183). However, more recent studies do not support this early conclusion but instead find that age has no effect on peripheral aromatization (187,188,189). More importantly, the degree of obesity effects the efficiency of aromatization. In both young and postmenopausal women, excess weight (240-430 lbs) increases conversion as compared to young and postmenpausal non-obese women (188,190,191). Thus, the "estrone hypothesis" is based on the observed association between conditions increasing peripheral aromatization (age and obesity) and endometrial carcinoma with the assumption that endometrial estrogenic stimulation unrelieved by progesterone will promote cancer growth.

To provide evidence in support of this hypothesis, investigators have compared the conversion constant of Δ to E_1 (190), the production rate of E_1 (192) and the serum concentrations of E_1 and E_2 (193,194) in postmenopausal women with and without endometrial carcinoma. The conversion of Δ to E_1, also was compared in groups of ovulatory and anovulatory (polycystic ovarian disease) young women (191). The conclusion from these studies was that, if comparisons are made within equal age and equal weight groups, there is no evidence for a difference in these parameters between normal women and women who have ovarian or uterine pathology.

One of the reasons for the higher plasma concentrations of free E_2 in obese women is the unexplained decrease in the concentration of plasma testosterone-estradiol-binding globulin (TeBG) found in obese people (195,196). This decrease in TeBG occurs equally in obese women with and without endometrial carcinoma (197,198).

The hypothesis of unopposed estrogenic stimulation promoting endometrial carcinoma someday may be proven in women who are "predisposed" to endometrial cancer. Yet there is also the possibility that the source of the stimulation of the endometrium is not estrogenic. As Siiteri has said recently (199), "how estrogens promote cancer is still not known. The generally low incidence of endometrial cancer even in those individuals receiving large amounts of exogenous estrogen argues against a mechanism whereby the hormones or their metabolites are carcinogenic. Indeed, that estrogen consumption by women increases their risk of the development of endometrial cancer is still a controversial issue."

An argument can be made against estrone as the source of uterine stimulation using as evidence the endometrial metabolism of estrogens. For estrone to stimulate the tissue efficiently, it must be reduced to E_2 since E_1 itself is not receptor-bound in human endometrial nuclei under physiologic conditions (200,201). The enzyme responsible for the reduction of E_1 to E_2, 17β hydroxysteroid dehydrogenase, preferentially oxidizes E_2 to E_1 (107), and as this enzyme is active in well-differentiated carcinomas (202) (the type most frequently found in pre- and postmenopausal endometrial cancers), it would serve as an inactivating influence, oxidizing E_2 to E_1 instead of

the reverse reaction.

Essential to the "estrone hypothesis" of endometrial cancer is the conversion of androgens to estrogens. However, this conversion may not be essential for a theory on the etiology of endometrial carcinoma. As already mentioned, hyperandrogenic pathological states are associated with a high incidence of endometrial hyperplasia and cancer. These disease states are all recognized risk factors for endometrial carcinoma (167), in young women, polycystic ovarian disease and Stein-Leventhal syndrome and in older women, ovarian tumors. The major risk factor, obesity, is not usually considered a physical condition with higher than normal androgen concentrations. Yet, the obese postmenopausal woman's plasma concentration of free T and DHT can be expected to be higher than the concentration in non-obese postmenopausal women because of the lower TeBG levels associated with obesity. In addition, as TeBG concentrations decrease, the increased free testosterone further inhibits the liver production of TeBG, thus serving to amplify its own free plasma concentration (203). Thus, all these risk factors for both age groups share in common an excess of circulating androgens.

Accepting the fact that women with elevated circulating androgens are at greater risk for developing endometrial cancer than are women with normal androgen concentrations, it should be considered on the basis of the metabolic pattern of androgen metabolism, which I have described in this thesis, that excessive endometrially produced DHT could act as a growth stimulant through the DHT receptor found in normal and cancerous endometrial tissue (204), and when this

stimulation reached a critical level, endometrial cancer could develop in women who are "predisposed" to it. The concentration of DHT needed to stimulate cancer growth need not be unusually large as the average concentration of DHT in carcinomatous prostatic tissue (4.1 mg/g) is only twice that found in normal prostatic tissue (2.1 ng/g) (205). Thus, endometrial tissue which concentrates some plasma DHT precursors (106) could contain sufficient substrate for the intracellular production of DHT in amounts capable of promoting uterine hyperplasia and endometrial cancer in "susceptible" women. In this way, DHT rather than E_1 could be the stimulating agent in some endometrial pathology.

In conclusion, it is unknown whether androgens have a physiological function in endometrial tissue. In those animals studied, including primates, large pharmacologic doses of androgens have "estrogenic effects" upon the uterus, whereas smaller pharmacologic doses have "antiestrogenic effects." The only information available on the human uterus concerning androgen action relates to the effects of short-term testosterone therapy and these effects are "antiestrogenic."

The effects of long-term androgen action upon the human uterus can be surmised from observing the uterus in pathological conditions of hyperandrogenism. One observation that has been made is the close association between these hyperandrogenic states and endometrial cancer. This association has been explained by the "estrone hypothesis", which concludes that the excess circulating androgens are peripherally aromatized, primarily to estrone and this estrone

causes uterine hyperplasia which can lead to endometrial carcinoma in women "predisposed" to endometrial cancer.

In the light of the experimental data on androgen metabolism in the human uterus presented in this thesis and earlier established information about uterine estrogen metabolism, it can be considered that the stimulator in the uterus of hyperplasia and endometrial carcinoma is not estrone from the peripheral aromatization of excessive circulating androstenedione acting through the estradiol receptor but endometrially produced DHT acting through its own receptor. As this thesis describes, both testosterone and androstenedione are 5α reduced by endometrial tissue leading to an intracellular pool of physiologically active DHT. Albeit, this pool of DHT may be small normally and its physiological function goes unrecognized in normal cycling women, when normal cycling has ceased and this pool of DHT enlarges, due to the increased availability to the uterus of androgen precursors, the intracellularly produced DHT may have a stimulatory effect upon uterine growth and may cause hyperplasia. Because of the information presented here concerning androgen metabolism in endometrial tissue, I believe a plausable argument can be made for a direct androgen involvement (at the endometrial tissue level) in the genesis of endometrial hyperplasia and cancer.

(b) Metabolism of testosterone by endometrial cancer cells in culture

(i) Purpose

In order to broaden this study of androgen metabolism in endometrial tissue (and to determine whether cancerous tissue might metabolize androgens in a different pattern than normal tissue), several batch incubation experiments were carried out with ^{3}H-testosterone and HEC-1A and HEC-1B cells, which are two cell lines that are maintained in culture in this laboratory. These cell lines arose from an endometrial adenocarcinoma (206). In addition, a unique type of cells, designated XY endometrial cells, were incubated with ^{3}H-T. These cells were derived from an epithelial carcinoma of the uterus of a phenotypic female with an XY genotype. They grew rapidly to confluence and were subjected to 12 passages during a 15 week period before they were used for metabolic studies. It is accepted that no comparison can be made of the metabolism of androgens by fresh minced tissue in superfusion and cells maintained in culture.

(ii) Experimental Design: Material and Methods

There were four experiments using a batch incubation procedure with human endometrial cancer cells in culture. The cancer cell types were the XY endometrial cells described earlier and two endometrial adenocarcinoma cell lines, a diploid cell HEC-1A and a predominately tetraploid cell HEC-1B.

These cells were grown in HAM F-10 medium (Flow Laboratories) containing 10% calf serum for the HEC cells and 10% fetal calf serum for the XY endometrial cells. The medium also contained antibiotics, 5 mg/ml glucose and 10 µg/ml insulin. Immediately preceding the

incubation when the cultures were confluent, cells were removed from the petri dishes with a buffered solution of 0.05% Trypsin and 0.02% EDTA (Flow Laboratories) pelleted, resuspended in Hank's balanced salt solution (Ca^{++}, Mg^{++}-free) (HBSS, GIBCO) repelleted and finally for the experiment suspended in Minimum Essential Medium (MEM), (Flow Laboratories) containing either [1β,2β-^{3}H]-testosterone (S.A. 40 Ci/mmol) or [7-^{3}H]-testosterone (S.A.= 25 Ci/mmol) dissolved in methanol to a concentration of less than 2%.

Table 20 contains the experimental conditions of each incubation. The first two experiments (M31 and A16) with HEC-1A and HEC-1B cells containing 437 and 811 pmol of ^{3}H-T, respectively, in 5 ml of incubation medium lasted for 6 and 17 hours. The final two experiments (J18 and A12) with HEC-1B cells containing 704 and 319 pmol of ^{3}H-T were for 6 hours in 5 ml of medium and 3 hours in 2 ml of medium, respectively. In the last experiment (A12), the XY endometrial cells were treated in the same manner as the HEC-1B cells. Control buffer with an equal concentration of ^{3}H-T as in the incubations was processed along with the cultured cells in three of the experiments. The three hour incubation was carried out in a shaking bath (GCA/Precision Scientific) at 37 C, while the longer incubations were performed in the culture room incubator (National, Weinicke Company).

The incubations were terminated after the addition of steroid carriers for T, Δ, Adione, androsterone and 5α-androstane-3α,17β-diol (500 μg) by decantation of the medium, as shown in Flow Chart 3. The cells were then homogenized in methanol, centrifuged and the pellet was saved for DNA determination according to Burton (207).

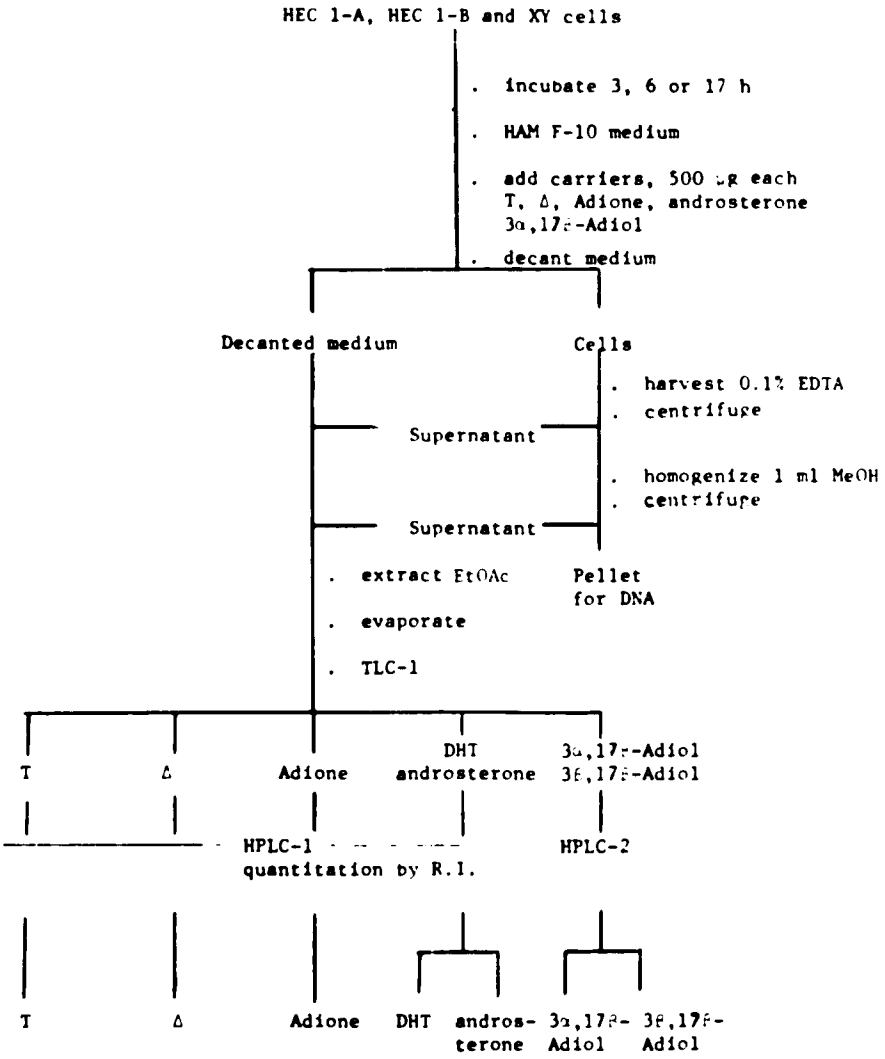
Table 20

Experimental Conditions: Cancer Cell Culture

Exp. #	Cell type	Number cells per dish	Total DNA per dish (µg)	Medium (ml)	Concentration ^3H_T substrate (nm)	Incubation time (h)
M31	HEC 1-A	17×10^6	152	5	1.8	6
	HEC 1-B	14×10^6	217	5	1.8	6
A16	HEC 1-A	8×10^6	216	5	3.2	17
	HEC 1-B	10×10^6	345	5	3.2	17
J18	HEC 1-B	10×10^6	125	5	2.8	6
A12	XY	5×10^6	120	2	7.0	3
	HEC 1-B	9×10^6	233	2	7.0	3

Flow Chart 3

Experimental Design; Cancer Cell Incubations



crystallization after addition of 5 mg carriers
$^{3}H/^{14}C$ ratios and specific activity by weighing

The supernatant was added to the decanted medium and the labeled steroids were extracted with ethyl acetate. Following this extraction, the steroids were separated, purified, quantitated and crystallized, using the same procedure as for T and Δ superfusion experiments.

(iii) Results

The calculated results from the HEC-1A, HEC-1B and XY endometrial cell incubation experiments with ^{3}H-T can be found on Table 21, while the crystallization data from these experiments is on Table 22. All three of these cell types actively metabolize testosterone.

HEC-1A and HEC-1B

Throughout the incubations with either the HEC-1A and HEC-1B cells whether for 3, 6 or 17 hours, the major metabolite was 3α,17β-Adiol. It comprised up to 61% of all the metabolites recovered from the ^{3}H-T. The other isolated metabolites, Δ, DHT and androsterone, were found in varying lesser amounts with Adione being recovered least. In the 17 hour incubation, androsterone was the principal metabolite recovered, probably because a 3α,17β-Adiol carrier was not added after this incubation. Also isolated from these incubation experiments, along with 3α,17β-Adiol was 5α-androstane-3β,17β-diol (3β,17β-Adiol). There was no carrier added before extraction for this compound but it was found during the process of purifying 3α,17β-Adiol by HPLC on a reverse phase column, using acetonitrile and water (v/v 50:50). The amount of 3β,17β-Adiol produced by the HEC cells is only 5 to 7% of the quantity of 3α,17β-Adiol reduced compound if one calculates that the recovery of both of these compounds initially isolated together after TLC is equal. Thus, the pattern of

Table 21

Experimental Results: Cancer Cell Culture

Cell type	Metabolite	Concentration pmol/mg DNA/nmol ^{3}H-T added				% of total metabolite isolated from ^{3}H-T			
		3h	6h		17h	3h	6h		17h
		(A12)	(J18)	(M31)	(A16)	(A12)	(J18)	(M31)	(A16)
HEC 1-B	Δ	47	193	52	23	13	35	18	19
	DHT	51	159	93	44	14	30	33	36
	Adione	3	10	3	2	1	2	1	2
	Androsterone	36	56	16	54	11	10	6	43
	3α,17β-Adiol	187	129	120		61	24	42	
HEC 1-A	Δ			137	92			24	40
	DHT			87	26			16	11
	Adione			4	3			1	1
	Androsterone			72	108			13	47
	3α,17β-Adiol			172				45	
XY	Δ	690				59			
	DHT	154				16			
	Adione	133				20			
	Androsterone	39				4			
	3α,17β-Adiol	7				1			

Table 22

Isotopic Data from Crystallizations of Androgens Isolated from Cancer Cell Incubation

Cell type (Exp. #)	Compound	Specific activity of steroid (weight of crystals, mg)					
		C-1	C-2	C-3	ML-1	ML-2	ML-3
HEC 1-A (M31)	T	1×10^6 (1.0)	1×10^6 (1.1)	1×10^6 (1.8)	1.4×10^6 (0.5)	1.4×10^6 (0.07)	1.5×10^6 (0.05)
	Δ	44,000 (0.9)	42,400 (0.9)	41,000 (1.2)	42,400 (0.2)	39,600 (0.08)	42,500 (0.08)
	DHT	39,400 (1.0)	34,300 (1.1)	27,500 (2.0)	34,700 (0.2)	27,500 (0.02)	
	Adione	1200 (1.0)	1100 (1.0)	900 (0.9)	1400 (0.4)		
	Androsterone	29,900 (1.0)	29,800 (1.0)	26,100 (1.2)	29,500 (0.2)		30,500 (0.03)
	3α,17β-Adiol	100,000 (0.5)	86,000 (0.7)		97,000 (1.9)	161,000 (0.01)	
	3β,17β-Adiol	4100 (0.9)	5100 (0.9)	5300 (1.7)	29,700 (0.1)		
HEC 1-B (M31)	T	1×10^6 (0.9)	1×10^6 (0.9)	1×10^6 (0.8)		1.5×10^6 (0.05)	1.5×10^6 (0.03)
	Δ	28,700 (1.0)	23,700 (1.0)	25,300 (1.3)	25,100 (0.2)	28,800 (0.3)	24,500 (0.1)
	DHT	57,200 (1.0)	49,700 (1.0)	54,700 (1.1)	58,900 (0.1)		49,700 (0.04)
	Adione	1300 (1.0)	1300 (1.0)	1000 (1.2)	1800 (0.2)		1100 (0.05)
	Androsterone	9800 (1.0)	9200 (1.0)	9500 (0.9)	12,900 (0.5)		15,400 (0.01)
	3α,17β-Adiol	45,100 (1.0)	46,400 (1.0)	45,000 (2.0)	35,000 (0.03)	39,000 (0.2)	45,000 (0.1)
	3β,17β-Adiol	5600 (1.0)	3000 (0.9)	3200 (1.3)	41,800 (0.2)	49,200 (0.02)	22,000 (0.07)

Table 22 - continued

	T	607,000	623,000	612,000	661,000	648,000	541,000
HEC 1-A		(1.0)	(1.1)	(1.5)	(0.4)	(0.3)	(0.07)
(A16)	Δ	54,300	52,800	53,000	53,600		48,400
		(1.1)	(0.9)	(1.1)	(0.1)		(0.05)
	DHT	13,300	12,900	12,700	51,400	19,200	
		(1.0)	(1.0)	(1.4)	(0.04)	(0.02)	(0.05)
	Adione	1400	1100	1200	1600	1400	3000
		(1.1)	(1.0)	(0.5)	(0.5)	(0.2)	(0.02)
	Androsterone	51,500	49,900	46,900	56,300	56,400	50,300
		(1.0)	(0.9)	(1.0)	(0.2)	(0.2)	(0.07)
	T	425,000	617,000	566,000	628,000	591,000	630,000
HEC 1-B		(1.1)	(1.1)	(1.2)	(0.6)	(0.08)	(0.05)
(A16)	Δ	21,300	20,600	20,100	26,000	25,300	23,500
		(1.0)	(1.0)	(0.9)	(0.2)	(0.2)	(0.05)
	DHT	31,300	30,900	28,300	38,700	35,100	38,600
		(0.9)	(1.1)	(0.9)	(0.2)	(0.06)	(0.03)
	Adione	2000	1900	1900	2200	2100	2200
		(0.9)	(0.8)	(0.4)	(0.8)	(0.2)	(0.06)
	Androsterone	48,800	48,500	44,000	55,900	52,200	45,300
		(1.0)	(0.8)	(0.9)	(0.6)	(0.09)	(0.03)
	T	1.2×10^6	1.3×10^6	1.2×10^6	1.4×10^6	1.2×10^6	1.3×10^6
HEC 1-B		(1.0)	(0.7)	(0.4)	(0.3)	(0.2)	(0.3)
(J18)	Δ	44,600	64,200	62,300	64,100	63,100	65,500
		(1.0)	(1.1)	(1.1)	(0.4)	(0.01)	(0.3)
	DHT	37,900	38,100	37,400		52,500	37,300
		(1.1)	(1.0)	(2.6)		(0.03)	(0.05)
	Adione	3000	3200	2700	3300	3000	2700
		(1.1)	(1.2)	(1.5)	(0.1)	(0.2)	(0.08)
	Androsterone	13,000	13,000	13,000	14,500	12,800	13,300
		(1.0)	(1.3)	(1.3)	(0.3)	(0.01)	(0.08)
	3α,17β-Adiol	36,500	36,700	35,600	32,200		39,100
		(1.0)	(1.1)	(2.0)	(0.1)		(0.2)
	3β,17β-Adiol	3600	2400	2600	28,000	8400	7500
		(1.0)	(1.0)	(0.7)	(0.9)	(0.1)	(0.02)
	T	535,000	513,000	519,000	498,000	496,000	531,000
		(1.0)	(1.1)	(1.4)	(0.5)	(0.1)	(0.02)
XY	Δ	71,800	69,800	70,100	76,500		
(A12)		(1.1)	(1.2)	(1.6)	(0.1)		

Table 22 - continued

	DHT	13,400	11,900	11,000	23,500	15,100	10,200
		(1.1)	(1.0)	(2.5)	(0.07)	(0.2)	(0.1)
	Adione	14,800	14,100	14,200	15,000		14,000
		(1.1)	(1.1)	(1.3)	(0.8)		(0.1)
	Androsterone	3300	2300	2700	5900	3900	4800
		(1.0)	(1.1)	(1.5)	(0.2)	(0.03)	(0.2)
	3α,17β-Adiol	1100	970	910	1640	2300	1650
		(0.9)	(1.0)	(1.9)	(0.2)	(0.06)	(0.2)
	3β,17β-Adiol	980	370	610	11,000	1500	1300
		(1.0)	(0.5)	(0.5)	(0.3)	(1.1)	(0.05)
	T	183,000	179,000	179,000	177,000	171,000	
		(1.0)	(1.0)	(1.8)	(0.3)	(0.2)	
HEC 1-B	Δ	11,100	9500	9800	11,200		10,900
(A12)		(1.1)	(1.1)	(1.8)	(0.6)		(0.2)
	DHT	11,300	10,200	10,300	14,000	15,000	11,400
		(1.1)	(1.1)	(1.7)	(0.03)	(0.09)	(0.09)
	Adione	640	640	630	650		
		(1.0)	(1.0)	(1.3)	(0.9)		
	Androsterone	7100	6700	6800	6600	6700	6800
		(1.1)	(1.0)	(1.4)	(0.1)	(0.2)	(0.1)
	3α,17β-Adiol	50,300	35,400		47,800	50,500	
		(0.8)	(0.2)		(2.4)	(0.7)	
	3β,17β-Adiol	3800	2500		15,000	3900	
		(0.9)	(0.3)		(1.3)	(0.5)	

testosterone metabolism by the HEC cells is principally reductive (note the small amount of Adione recovered) except for some oxidation of testosterone to androstenedione.

XY Endometrial Cells

The XY endometrial cells in comparison produced very little 3β,17β-Adiol from testosterone. It being the least metabolite relative to the total isolated while Δ was the major metabolite isolated. Androstenedione made up 59% of all of the testosterone metabolites recovered after 3 hours of incubation. Adione and DHT were found in similar but lesser amounts. Thus, the pattern of testosterone metabolism for these cancer cells is more like normal endometrium, which actively oxidizes T to Δ, while 5α reducing some of both the T and Δ to DHT and Adione.

(iv) Conclusions

These transformed endometrial cells in culture contain the same enzymes for the metabolism of androgens as do normal endometrial cells, 17β hydroxysteroid dehydrogenase, 3α hydroxysteroid dehydrogenase and 5α reductase. The pattern of metabolism of the XY endometrial cells seems to be very similar to that of normal endometrium with 17β hydroxysteroid dehydrogenase actively oxidizing T to Δ, whereas in the HEC cell, the dehydrogenases seem to favor reduction as evidenced by the production of large amounts of 3α,17β-Adiol and small amounts of Adione. The difference between the patterns of metabolism of the cell lines and the XY endometrial cells may be related to the fact that the HEC cells are established cell lines, whereas the XY cells, like normal tissue in culture, did not divide

indefinitely but after the 10 months in culture and 49 passages ceased to grow and could not be maintained.

The formation of 3α,17β-Adiols by cancer cells has been described earlier after incubation of carcinomatous uterine tissue. In addition to my findings, Unterberger et al concluded that human endometrial carcinomas produce statistically significantly more 3α,17β-Adiol than normal endometrium (208) and Per Stenstad et al reported that NHIK 3025 cells (human uterine cervical carcinoma cell line) produced between 2 1/2 and 50 times more 3α,17β-Adiol from ^{3}H-T than any other isolated metabolite (209).

The isolation of 3β,17β-Adiol from the HEC-1 cell incubations is noteworthy because this compound has been found to inhibit in a concentration-dependent manner the initial rate of formation of the 17β-estradiol-receptor complex in human endometrium (210) though unlike 5-androstene-3β,17β- diol (211, 212), does not compete with estradiol for binding to the uterine estradiol receptor.

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