Title
Steroid-receptor interaction and effects of C-7 substituents on the biological properties of androgens

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STEROID-RECEPTOR INTERACTION AND EFFECTS OF C-7 SUBSTITUENTS ON THE BIOLOGICAL PROPERTIES OF ANDROGENS

by

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DISSERTATION

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STEROID-RECEPTOR INTERACTION AND EFFECTS OF C-7 SUBSTITUENTS ON THE BIOLOGICAL PROPERTIES OF ANDROGENS

by

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ABSTRACT

Since elimination of androgenic activity is essential for the development of drugs useful in the treatment of female breast cancer, one approach has been to look for the separation of biological activities by chemical modification of the steroid molecule. In the present study, two compounds, namely 7β-methyl-5α-dihydrotestosterone and 7β-methyl-2-thia-A-nor-5α-androstan-17β-ol, were designed on the basis of findings that the introduction of 7β-methyl group into testosterone (e.g. Calusterone) increases the anti-tumor activity, while decreasing the androgenic activity, and that the enhancing groups known to be useful in the carbocyclic steroid series could be introduced into the heterocyclic steroid series to give similar activity.

The preparation of 7β-methyl-5α-dihydrotestosterone and its 2-thia-A-nor analog was studied. 6-Dehydro-7-methyltestosterone was prepared in good yield by a reported procedure. Catalytic hydrogenation afforded 7β-methyl-5α-dihydrotestosterone. Cleavage of A ring with CrO_3-HOAc gave diacid which was converted to dibromide by the modified Hunsdiecker reaction. By cyclization in the presence of Na_2S, the dibromide gave 7β-methyl-2-thia-A-nor-5α-androstan-17β-ol.

Biological evaluation showed that 7β-methyl-5α-dihydrotestosterone was only weakly androgenic, whereas 7β-methyl-2-thia-A-nor-5α-androstan-17β-ol was devoid of androgenic activity. These results are in good agreement with previous findings that 7α-methyl substitution increases both androgenic and anabolic potencies, whereas 7β-methyl substitution decreases both activities to a very large degree. At the dose level of 10 mg/Kg/day both compounds were non-toxic and produced no significant tumor-inhibitory effects. However, the final tumor size in animals treated with 7β-methyl-2-thia-A-nor-

5α-androstan-17β-ol was significantly reduced. Since toxic effects were not evident and only one dose level was used, there is little question that larger doses could be safely tolerated. Based upon the limited results of this study, the determination of the tumor-inhibitory action of both compounds would have to be carried out at dose levels of about 50 mg/Kg/day.

The effect of nonradioactive steroids on retention of radioactive dihydrotestosterone by ventral prostate in vitro and in vivo was studied. Efforts were made to correlate chemical structure with receptor binding affinity and androgenic activity. The data in the present study, coupled with previous findings, suggest that both receptor binding affinity and intrinsic activity are involved in determining the potency of 7-methyl substituted androgens and both β-face and thickness of steroid molecule play an important role in determining the intrinsic activity and the binding affinity. The result from in vivo competition experiment indicates that the nonandrogenic action of 7β-methyl-2-thia-A-nor-5α-androstan-17β-ol is due to lack of intrinsic activity. Thus, like cyproterone, this compound largely inhibited the uptake of radioactive dihydrotestosterone by ventral prostate in vivo and in vitro. However, in the anti-androgenic anabolic tests, this compound did not antagonize the androgenic action of 5α-dihydrotestosterone on the ventral prostate and the seminal vesicles. This may be attributed to the short half-life of the compound.

The previous findings regarding three separate binding sites in the ventral prostate for 5α-dihydrotestosterone, 5α-androstan-17β-ol, and 5α-androst-2-en-17β-ol was finally reinvestigated. Evidence strongly indicates that the previous findings were made from the incorrect interpretation of the inhibition of radioactivity uptake by ventral prostate. However, the question of the number of separate binding sites remains to be answered.

DEDICATION

To Sugi

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To Dr. Manfred E. Wolff, my advisor, for his guidance, encouragement, and patience throughout my study.

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To the fellow graduate students in 1136-S for their companionship.

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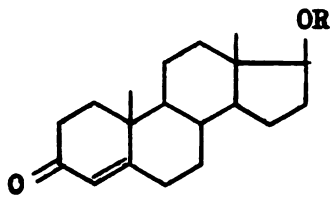
PART I

INTRODUCTION

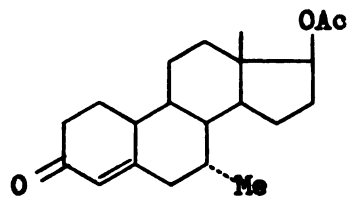
Female breast cancer is one of the most prevalent forms of human cancer and is the leading cause of cancer deaths among women in the United States. The conventional primary treatment of breast cancer is mastectomy, frequently followed by postoperative irradiation of surrounding areas as adjuvant therapy, but where advanced inoperable mammary cancer is present, or disseminated cancer is beyond the reach of surgical or radiological treatment, the therapy is based on alteration of the hormonal status of the patient. This can be accomplished either by ablation of the ovaries or the adrenals or by administration of their hormonal secretions such as estrogens, progestins, corticosteroids, and androgens. Hormonal therapy is based on the concept that some cancer cells derived from hormone-responsive organs, notably the mammary gland and the prostate, are not completely autonomous, but often retain some of the hormonal requirements of their normal counterparts for at least some part of their life. By changing the hormonal environment of such tumors it is possible to alter, to some degree, the course of the neoplastic process.

Earlier studies had suggested a direct relationship between androgenic potency and activity against human breast cancer. In the past, the androgen most widely reported was testosterone propionate, and the prolonged therapy necessary for the control of breast cancer was associated, in the case of this agent, with a considerable degree of masculinization. In the last decade, equally effective, but less masculinizing, androgens have been introduced. For example, the powerful androgenic and anabolic agent, 7α-methyl-19-nortestosterone acetate, and less androgenic compounds such as 2α-methyl-5α-dihydrotestosterone propionate and D-homotestosterone propionate, as well as the completely nonandrogenic steroid, Δ^1-testolo-

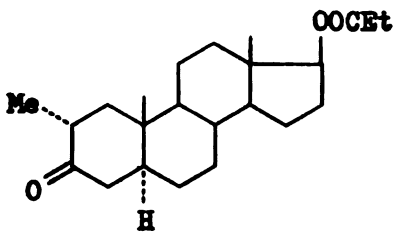


R = H, Testosterone

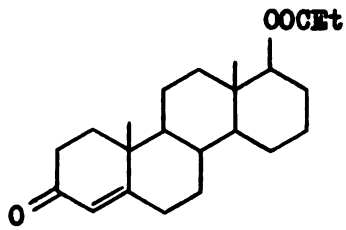
R = EtCO, Testosterone propionate



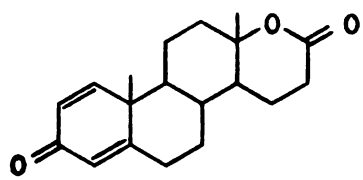
7α-Methyl-19-nortestosterone acetate



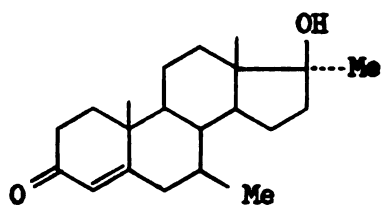
2α-Methyl-5α-dihydro-testosterone propionate



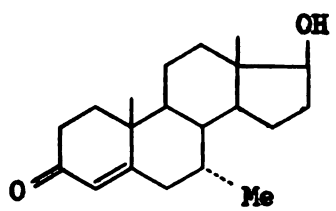
D-Homotestosterone propionate



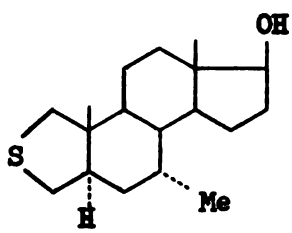
13α-Hydroxy-13,17-secoandrosta-1,4-dien-17-oic acid-3-one lactone (Δ^1-Testololactone)



7β,17α-Dimethyltestosterone (Calusterone)



7α-Methyltestosterone



7α-Methyl-2-thia-A-nor-5α-androstan-17β-ol

lactone, all elicited responses similar to that of testosterone propionate. In line with this, it has recently been found that 7β,17α-dimethyltestosterone (Calusterone), a very weak androgen, has higher anti-tumor efficacy than any other steroid hormone currently employed in the treatment of advanced female breast cancer.

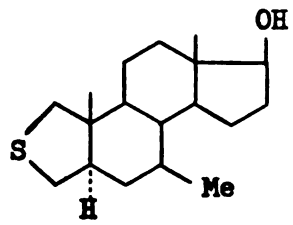
Since compounds with little or without androgenic activity are especially suitable for use in the treatment of female breast cancer, one approach has been to look for splits in biological activities by chemical modification of the steroid molecule. The rationale for this lies in the idea that minor chemical alterations of the steroid molecule may selectively increase certain features of biological activity of the parent compound with concomitant reduction in undesirable activities. It has been shown that the introduction of an enhancing group such as 7α-methyl group into a steroid molecule (e.g. 7α-methyltestosterone) increases the androgenic and anabolic potency of the parent compound. On the other hand, the introduction of 7β-methyl group into a steroid molecule (e.g. Calusterone) decreases both androgenic and anabolic activities, while increasing the anti-tumor activity. It also has been demonstrated that the enhancing groups known to be useful in the carbocyclic steroid series could be introduced into the heterocyclic steroid series to give similar activity. Thus, for example, the introduction of a 7α-methyl group into 2-thia-A-nor steroid molecule increases both androgenic and anabolic activities of the 2-thia-A-nor steroid. Similarly, one would expect that the introduction of a 7β-methyl group into the 2-thia-A-nor steroid molecule would increase the anti-tumor activity, while decreasing the androgenic activity. This rational basis was applied in the present study to develop potential new drugs useful in the treatment of advanced female breast cancer.

The work described in this thesis involved synthetic chemistry, evalu-

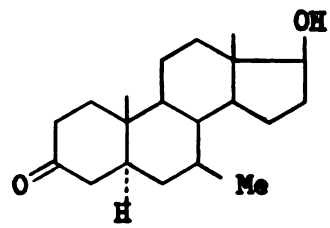
ation of biological data, steroid-receptor interaction, and examination of the relationship between chemical structure, biological activity, and receptor binding affinity. The first goal of the thesis was to synthesize 7β-methyl-5α-dihydrotestosterone and 7β-methyl-2-thia-A-nor-5α-androstan-17β-ol and to evaluate their biological activities.

The effect of C-7 substituents on the steroid-receptor interactions was next studied. It was carried out by incubation of minced ventral prostate of castrate rats with ^{3}H-5α-dihydrotestosterone and nonradioactive steroids or by injection of ^{3}H-5α-dihydrotestosterone and nonradioactive steroids into castrate rats. Efforts were made to explain the action of C-7 substituents at the receptor affinity-intrinsic activity level and to correlate chemical structure with receptor binding affinity and biological activity.

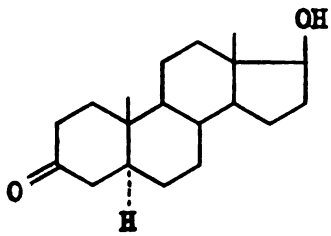
This work was finally extended to study the anti-androgenic action of antiandrogens as well as the type of receptors responsible for androgenic and anabolic activities. The question of whether there are three separate binding sites in the ventral prostate for 5α-dihydrotestosterone, 5α-androst-2-en-17β-ol, and 5α-androstan-17β-ol was discussed.



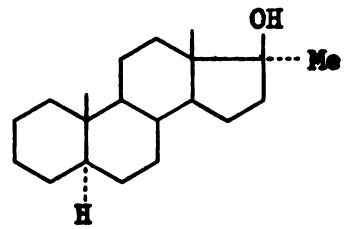
7β-Methyl-2-thia-A-nor-5α-androstan- 17β-ol



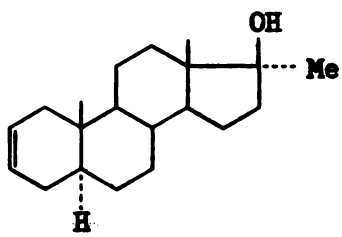
7β-Methyl-5α-dihydrotestosterone



5α-Dihydrotestosterone



17α-Methyl-5α-androstan-17β-ol



17α-Methyl-5α-androst-2-en-17β-ol

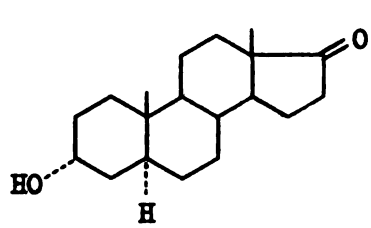
PART II

GENERAL CONSIDERATIONS

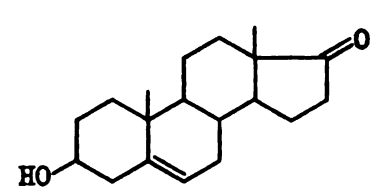
(A) Androgens and anabolic steroids.

Androgens are defined as a group of biologically active steroidal compounds characterized by their biological effects on the primary and secondary sex characteristics of the adult male or their vestigal remains in the female. These are: remarkable changes at puberty that transform the boy into a man, increased size of the phallus (penis or clitoris), growth and pigmentation of the scotum (or labia majora), development of the prostate and seminal vesicles, deepening of the voice, stimulation of the sebaceous secretion, and the appearance of sexual hair.

The isolation of the urinary principle with androgenic activity was first accomplished in 1931 by Butenandt (1), who obtained 15 mg of crystalline substance, named androsterone (2), from 15,000 liters of male urine, and in 1932 Butenandt proposed a structure formula that was later shown by synthesis to be correct (3). Further chemical investigations led to the isolation, in 1934 (4), of another weakly androgenic steroid hormone from male urine. At first called dehydroisoandrosterone, this substance was named dehydroepiandrosterone because of its ready chemical transformation and structural similarity to androsterone.



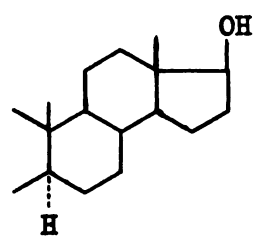
3α-Hydroxy-5α-androstan-17-one
(Androsterone)



3β-Hydroxyandrost-5-en-17-one
(Dehydroepiandrosterone)

Attention next focused on the testes as the real source of male sex hormone. Active testicular extracts were first prepared as early as 1927 by Loewe (5), using the mammalian seminal vesicle as a test. The testicular androgenic hormone, testosterone, was isolated in crystalline form by Laqueur and colleaques (6,7). This substance was nearly 10 times as active as androsterone in promoting comb growth in the capon, and about 70 times as potent in its action on the seminal vesicles of castrated rats. Shortly after this discovery the first chemical synthesis of testoaterone was reported by Butenandt and Hainsch (8) and quickly confirmed by Ruzicka (9, 10).

A great many other steroids with androgenic activity soon became known; some were isolated from ovarian and adrenal tissue as well as from the testis, and numerous analogs and derivatives were prepared. An androgenic-anabolic nonsteroid, namely 1,4-seco-2,3-bisnor-5α-androstan-17β-ol, was first reported in 1973 by Zanati and Wolff (11).



1,4-Seco-2,3-bisnor-5α-androstan-17β-ol

The androgens are secreted mainly by the testes, ovary, and adrenal cortex. Testosterone, probably the most potent naturally occuring testicular hormone, is formed by the Leydig cells of the testes. Like other steroid hormones, testosterone and the other androgens appear to be derived from cholesterol or its sulfate (12).

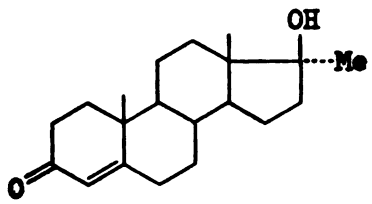
The anabolic action of androgens was first discovered in 1935 by

Kochakian and Murlin (13). Extracts of male urine injected into dogs not only had an androgenic effect but, in addition caused retention of nitrogen (anabolic effect). Since then, numerous investigations have led to the realization that steroid hormones have many potent effects on the body and minor chemical alterations of the steroid molecule may increase some of these effects without affecting others. After the anabolic properties of androgens were confirmed on both physical and pharmacological grounds (14), a great many synthetic androgens were prepared and tested in the search for compounds that might promote general body growth without having masculinizing effects. However, a complete dissociation of androgenic and anabolic effects has not yet been achieved, and even the degree of dissociation reached so far is a matter of debate. Some androgenic-anabolic steroids used in therapy are shown in Fig. 1.

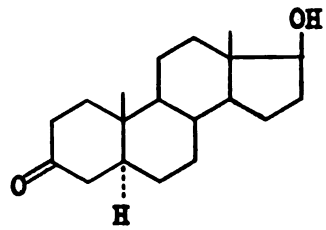
The main application of androgenic hormones is replacment therapy in cases of testicular deficiency or decreased testosterone production. They are used to treat hypogonadism, hypopituitarism, osteoporosis, menstrual disorders, refractory anemia, mammary cancer, and to promote anabolism. Their use as anabolic agents, although of much wider potential application, is still in the stage of exploration (15).

Unfortunately, untoward side effects of androgens limit the clinical use in humans. When used in women, all of the androgens carry the risk of causing masculinization. Undesirable effects on sexual and osseous development were noted in children on anabolic therapy. Both sexes may experience salt and subsequent water retention. Furthermore, certain 17α-alkylated androgens cause a type of liver damage, called cholestatic hepatitis (15). The biochemistry, mode of action, and therapeutic application of androgens has recently been reviewed (16).

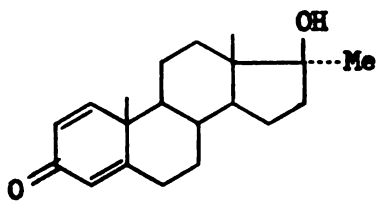
Fig. 1. Some androgenic-anabolic steroids used in therapy



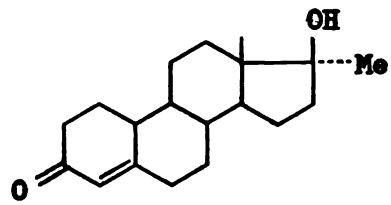
17α-Methyltestosterone
(Mesterone)



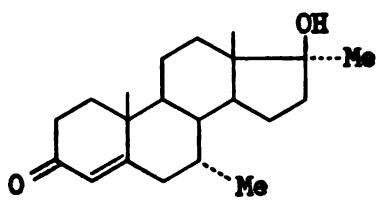
5α-Dihydrotestosterone
(Androstanolone)



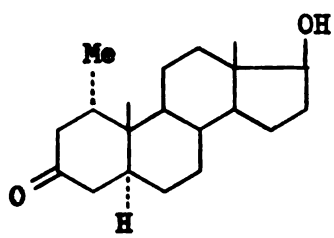
Methandrostenolone



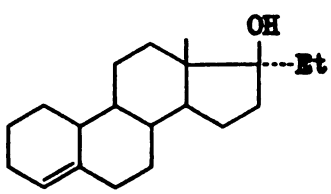
17α-Methylnortestosterone



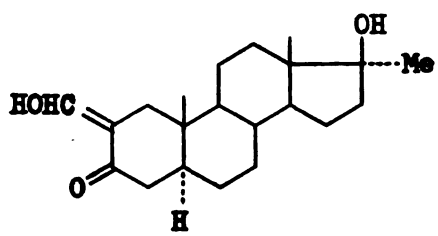
Bolasterone



Mesterolone

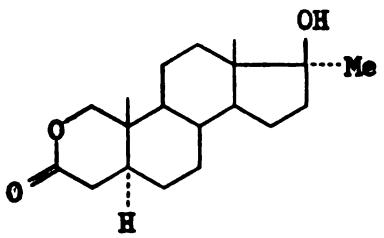


Ethylestrenol

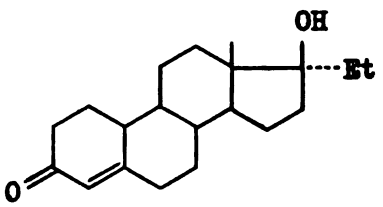


Oxymetholone

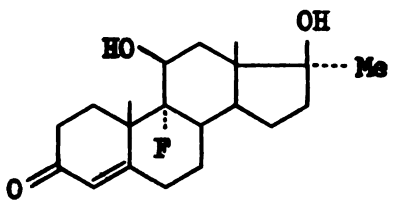
Fig. 1. (continued)



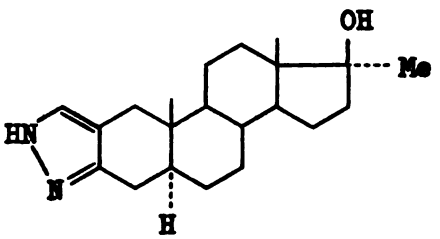
Oxandrolone
(Anavar)



Norethandrolone
(Nilevar)



Fluoxymesterone
(Halotestin)



Stanozolol
(Winstrol)

(B) Bioassay and potency.

The variety of methods of biological evaluation used to establish the androgenic properties of steroidal substances has been reviewed in detail by Dorfman (17). Classically, androgens were assayed by the capon comb growth method (18). Better parallelism with clinical effectiveness is given by assays in mammals, and the most widely used test depends upon the increase in weight of the seminal vesicles and the ventral prostate of the immature castrated male rat (19-22). The test compound is administered either intramuscularly or orally, and the weight of the target organs is compared with those of control animals.

To define the androgenic action of a steroid in terms of its effect on the secondary male cheracteristics is very vague since the secondary male characteristics are localized in many different tissues and vary considerably between species. The extent to which these different tissues are restored after the administration of androgens can differ widely among the various compounds. Therefore, for the purpose of pharmacological bioassay of steroid in mammals, it is generally agreed that the term "androgenic activity" is restricted to the effect of such compounds on organs directly involved in the production and transport of semen and that the comparison of the androgenic activity of various compounds can be made only on the basis of the comparison of their effects on the same organ in comparable test animals.

The methods employed to determine the anabolic or myotrophic properties of steroids have been reviewed (23). Generally these are based on an increase in nitrogen retention and/or muscle mass in various laboratory animals. The castrated male rat is currently the most widely used and most sensitive laboratory animal for nitrogen balance studies (24). Measurement of the excretion of nitrogen before and after giving the substance to be tested

provides an index to nitrogen-retaining potency (25). Although it is generally agreed that variation in urinary nitrogen excretion are related to an increase or decrease in protein synthesis, nitrogen balance assays are not without their limitations (26). This is partly due to the fact that such tests fail to describe the shifts in organ protein and measure only the overall status of nitrogen retention in the animal (27).

A systematic search for nonandrogenic anabolic steroids has made use of the growth of the easily accessible levator ani muscle of the castrated rat as a valuable index for measuring the anabolic activity of steroid hormones (20,22). By comparing the weight of this muscle to that of the ventral prostate or seminal vesicle, a ratio of anabolic to androgenic activity of a compound can be obtained. When this ratio is compared to that of testosterone propionate as a standard in parenteral tests, or to that of 17α-methyltestosterone when orally active compounds are being assayed, one can obtain a relative ratio of anabolic to androgenic activity of a steroid hormone. The limitations of the method using the levator ani response as an index for measuring the anabolic activity have been noted (28-30). Nevertheless, this is the only test in general use for rapid pharmacological screening and the correlation between the levator ani response and the nitrogen-retaining properties of a compound in general is remarkable (26).

(C) Antiandrogens

A search for compounds that might inhibit the action of androgen was doubtless promoted by clinical considerations. Treatment of cancer of the prostate was one of the earlier aims. These compounds would also be valuable in physiological and biochemical studies on hormonal interactions involving androgens and on the endocrine control of testicular maturation and function.

It has been known since 1938 that the actions of androgenic hormones are influenced by estrogens (31). Estrogens, in a restricted sense, may be regarded as anti-androgenic. They may directly antagonize selected androgenic actions, e.g. chick comb growth, but other anti-androgenic effects, e.g. decreased size of secondary sexual organs, are due to suppression of pituitary gonadotropin secretion which in turn results in decreased androgen secretion by the testes. Estrogens do not antagonize most of the actions of androgens on secondary sexual structures when both are administered to castrate animals. Therefore, the term "antiandrogen" should be applied to compounds that directly influence the receptors of all organs or systems of organs that are in any way functionally or morphologically androgen-dependent (32). They thus have no effect on the biosynthesis of testosterone.

In the search for more potent, orally active progestins, cyproterone acetate was encountered in 1963 by accidental observation of intrauterine feminization phenomena in animal experiments (33). It is 250 times as active as progesterone in the Clauberg test in rabbits when given subcutaneously and 1000 times as active when given by mouth. Pregnant rats treated with this substance apparently bore only female offspring. The development of the rudimentary male genitalia had been suppressed, the penis was underdeveloped and resembled a clitoris, the prostate was missing, and the testes were small and undescended. These changes were permanent (33).

In the male rat, cyproterone acetate causes atrophy of the seminal

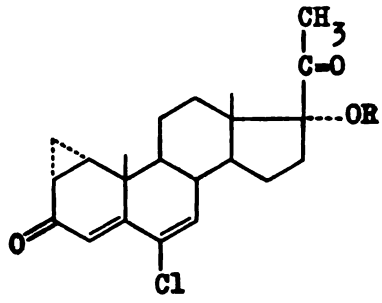
vesicles, prostate, levator ani muscle, and other androgen-responsive organs, as well as cellular changes in the pituitary resembling those of castration (34). The actions of testosterone in the castrated animal are inhibited in a direct dose-related manner, and only about 5 times as much antagonist as testosterone is needed to reduce the androgenic response by 50% (35). The complete inhibition of the effects of exogenous testosterone propionate on the seminal vesicles and prostate of rats requires 30 times as much cyproterone acetate, while 10 times as much is required to inhibit the effect on cock's combs.

Although cyproterone acetate is the most active antiandrogen so far encountered, closely related analogs are also active. The free alcohol is only about one third as active as the acetate and, surprisingly, is devoid of progestational activity, whereas the acetate is one of the most potent progestins known.

Since testosterone is presumably an important contributory cause of acne, this skin disorder should be alleviated by antiandrogens (36-38). Another interesting clinical possibility is the use of antiandrogens to control the libido of male with disturbed instincts, such as sexual offenders (39-41). Investigations on the inhibition of the partly androgen-dependent growth of carcinoma of the prostate by antiandrogens could also be important (42,43). They may also have a use as male contraceptives if side effects, such as impotency, can be eliminated.

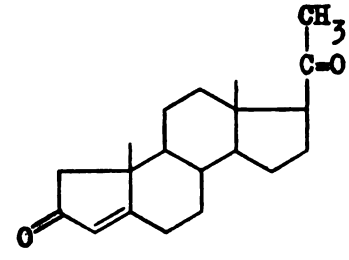
The antiandrogenic steroids that have been investigated belong mainly to the androstane and pregnane series. Some antiandrogenic compounds are shown in Fig. 2. The clinical effects and applications of antiandrogens has recently been reviewed (44).

Fig. 2. Some antiandrogenic compounds

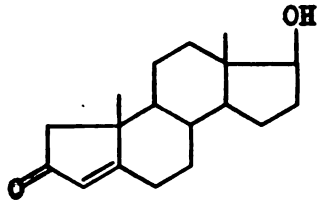


R = H, Cyproterone

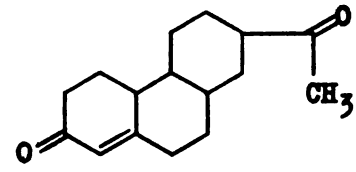
R = Ac, Cyproterone acetate



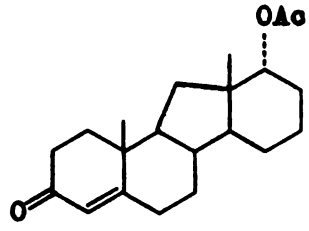
A-Nor-progesterone



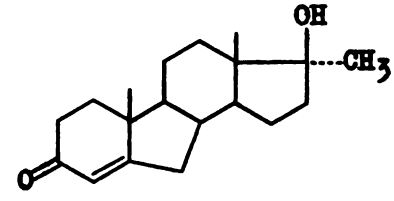
A-Nortestosterone



2-Acetyl-7-oxo-dodeca-hydrophenanthrene



C-Nor-D-homo-17α-epi-testosterone acetate



17α-Methyl-B-nortestosterone

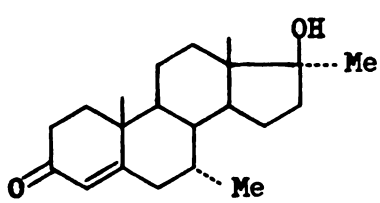
(D) Androgen therapy of breast cancer.

Cancer of certain organs or cells that are normally under hormonal regulation often can be treated with hormonal therapy. This is based on the concept that some cancer cells derived from a hormone-responsive organ, notably the mammary gland and the prostate, are not completely autonomous, but are subject to hormonal control, at least during some part of the life of the cancer. The conventional primary treatment of breast cancer is mastectomy, frequently followed by postoperative irradiation. However, treatment of advanced inoperable breast cancer or disseminated cancer beyond the reach of surgical or radiological treatment is based on alteration of the hormonal status of the patient. This can be accomplished either by administration of large doses of the sex hormones, such as androgens, estrogens, and progestins, or by removal of the sources of endogenous hormone production by oophorectomy (orchiectomy in the male), adrenalectomy, or hypophysectomy. The response to hormone therapy depends upon the age and menopausal status of the patient and the extent and nature of metastatic involvement. Treatment with hormones is indicated only in patients with disseminated disease (45).

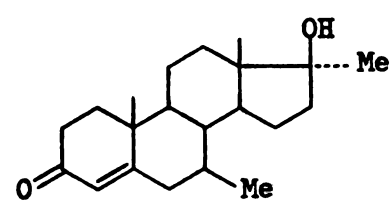
The androgens have been used as palliative agents in women at any stage of disseminated breast cancer, without relation to the menopause. Testosterone propionate, 100 mg IM 3 times weekly, results in subjective improvement characterized by euphoria and relief of pain in 20-25% of patients. Soft tissue metastases are less responsive to androgens, and regression occurs slowly. The duration of androgen-induced remissions is usually not over 6-8 months and seldom more than a year. Treatment with androgens must be continued for 10-12 weeks before its effect can be appraised. Further regression often follows discontinuation of therapy, so that it is desirable to wait several weeks before employing other methods of therapy. Unpleasant

and distressing side effects of androgen therapy include virilism, hirsutism, deepening of the voice, acne, flushing, sodium retention, and increased libido (45).

Because of the distressing masculinization often produced by androgenic therapy, one approach to improving treatment has been to look for splits in biological activity by chemical modification of the testosterone molecule. Numerous agents have been tested with this objective, and from these have come several effective but less androgenic compounds such as 2α-methyl-5α-dihydrotestosterone propionate (46) and the completely nonandrogenic steroid, Δ^1-testololactone (47). In 1959 Campbell and Babcock described the synthesis of some 7α- and 7β-methyl steroids (48). Bioassay showed that the presence of the 7α-methyl group appeared to increase androgenic and, notably, anabolic activity when compared to methyltestosterone (49). The 7β epimer, on the other hand, showed only weak biologic activity (50). Because of the possible correlation of anabolic and anti-tumor action (51), the 7α-methyl compound was tested in a protocol study of 25 women with objectively progressing disseminated breast cancer who had not previously been treated with hormonal agents (52). The reference standard against which this compound was randomly distributed was 2α-methyl-5α-dihydrotestosterone propionate. In five of 25 women treated with 7α,17α-dimethyltestosterone there was objective regression. They were also virilized but had no jaundice or other evidence of hepatoce-



7α,17α-Dimethyltestosterone
(Bolasterone)



7β,17α-Dimethyltestosterone
(Calusterone)

llular damage. Because the 7β epimer had less biologic activity when tested for usual hormonal effects, the possibility was considered that this analog might demonstrate the desired split of activities -- that is, retention or increase of anti-tumor efficacy with less of the undesirable hormonal and cholestatic effects. Although the screening against a battery of animal tumors showed no anti-tumor activity, the 7β epimer was accepted by the Cooperative Breast Cancer Group (53) for testing in carcinoma of the human breast because of their deep conviction that animal screens do not adequately predict activity against human female breast cancer. Using the strict criteria of the Cooperative Breast Cancer Group, 14 of the 22 women (64%) obtained objective regressions. The regressions obtained lasted for from 3 to 20 months with an average of 7 months for those who had response. This highly favorable objective regression rate in unfavorable subjects offered promise that Calusterone would be significantly more effective than any other sex steroid currently employed in the treatment of advanced breast carcinoma. The anti-tumor efficacy of all the usual hormones used for the treatment of this disease, when measured by group criteria, falls below 25%: (a) testosterone propionate, (b) diethylstilbestrol, (c) 2α-methyl-5α-dihydrotestosterone, (d) Δ^1-testololactone, (e) hydrocortisone + triiodothyronine (54), (f) fluoxymesterone (47), and others. By these criteria, the established chemotherapeutic agents, which are also more toxic, also fall in this same general range -- for example 5-fluorouracil, 23.1% (55) -- and the figures for chlorambucil, vincristine, methotrexate, and nitrogen mustard are all below this level.

However, Calusterone is not a cancer cure. Like other steroidal and chemotherapeutic agents it offers significant palliation and improvement in quality of life.

(E) Theory of androgen-receptor interaction.

The receptor theory of drug action implies that the pharmacological properties of a compound are dependent not only on the nature and properties of the constituent groups within the molecule but also in the way in which these groups are oriented.

Current theory of steroid-receptor interaction holds that steroids elicit their specific biological activity by interaction with a receptor protein. Because the molecules of steroids are relatively flat and rigid, their interaction with receptors can occur on the α-face (bottom side) or β-face (top side) of the steroid molecule. To explain the mode of action of androgens, Ringold (56) systematically examined the androgenic action of α- and β-alkyl- or halo-substituted androstanes and reached the conclusion that the interaction of androgens with a receptor to produce a classical androgenic response is on the α-face of the androgen molecule. This postulation was further supported by Zaffaroni (57), who studied the effects of alkyl and electronegative group substitutions on the activity of androgenic-anabolic steroids.

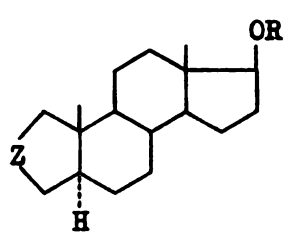
Bush (58), however, disagreed with Ringold's theory. Bush's main criticism of Ringold's theory centered around the uncertainty of the effects of 17α-substituents. As Bush remarked, "the 17α-methyl group, while conferring oral activity on a typical C-19 17β-ol, reduces androgenic potency by parenteral routes while leaving levator activity intact." Larger 17α-alkyl groups reduce or abolish androgenic activity, though most clinical workers have found that androgenic activity is still present. He also suggested that the 17β-hydroxyl group plays a specific role in the association of androgen with the receptors for levator and androgenic activity, since all active androgens possess a 17β-hydroxyl group or else a group which can be converted to it (17-ketone or 17α-hydroxyl). In addition, he called atten-

tion to the fact that in androgens substitutions and modifications in ring A have much more complicated and striking effects than in other classes of steroids. According to his data, Bush outlined the broader concept that changes in the configuration of the upper half of ring C and ring D have a far greater effect on all types of biological activity of a steroid than many changes in the configuration of rings A and B. Later, Bush (59) suggested that in the case of androgens, probably, but not certainly "the upper and β-sided surface of the molecule was responsible for conferring specificity on the steroid-receptor association" and the α-surface of the steroid is not involved in a specific interaction with the receptor, since 17α-methyl and 17α-ethyl substitutions already bring about considerable distortion of the molecular surface of the steroid.

In contrast to the conclusion of Ringold, Wolff and Jen (60) suggested that in the steroid-receptor complex the steroid is in contact with the receptor surface in two discrete areas: the β-face of rings A, B, and C, and the α-face of ring D. They proposed that the two principal binding sites are the A-ring, where a π-bond is formed, and the 17β-function, which can be attached by any of several types of nonbonded interactions. The remaining areas in contact with the receptor would form ordinary hydrophobic bonds or van der Waals bonds. Wolff et al. claimed that the effect of the steroid is to induce a conformational change in the receptor, since no chemical reaction as such takes place. Although these workers based their conclusion mainly on the evidance that introduction of more bulky substituents (CN, CHO, CHOH) in place of the C-19 methyl group decreased activity, this concept received further support when it was found that (a) 19-methyltestosterone was inactive (61) (Ringold predicted that this compound would be active), and (b) 7α-methyl (62) and C-13 angular ethyl (63) analogs display high androgenic activity. Crystallographic measurements of estriol seem to give

further support to Wolff's theory (64).

By varying the electron density pattern in and around ring A, Bowers et al. (65) concluded that a high electron density at C-2 and/or C-3 in 17β-hydroxyandrostane is a factor strongly promoting high myotrophic activity. This condition may be satisfied by a C-3 carbonyl or oxidizable C-3 hydroxyl group. It is possible that C-3 ketone may be active primarily as enols or enolate anions where a Δ^2 π-bond is present. Bowers also postulated that introduction of more than one sp^2-hybridized carbon atom into ring A results in a pronounced flattening of the ring from a cyclohexane chair form to a more planar conformation in which the steroid may be better able to rest on a receptor surface with a concomitant increase in the degree of orbital overlap. On the basis of biological evaluation of 11 steroids having cyclopropane, ethylene oxide, or spirooxiranyl rings fused to C-2 and C-3, Wolff et al. (66) also suggested a similar requirement for androgenic activity. However, the importance for androgenic activity of sp^2 atoms at C-2 and/or C-3 of steroids could be due to the steric characteristic of the sp^2 center, to the electronic nature of such atoms, or jointly, to both effects. Later, Wolff and Zanati (67) described the preparation of oxa-, selena-, tellurio-, and related steroids as probes of steroid structural requirements. The data from the pharmacological testing showed that selena- and thia-steroids, (d) and (b), have comparable activity but oxa-, sulfoxide, and sulfone derivatives, (a), (g), and (h), are inactive. Therefore, thia-steroid (b) is



a. Z=O, R=H
b. Z=S, R=H
c. Z=S, R=Ac
d. Z=Se, R=H
e. Z=Te, R=H
f. Z=S-S, R=Ac
g. Z= SO, R=Ac
h. $Z=SO_2$, R=Ac
i. $Z=CH_2CH_2CO$, R=H

almost certainly active as such, and not as its most likely sulfoxide and sulfone metabolites, (g) and (h) respectively. The covalent radii (68) of selenium (1.17 Å) and sulfur (1.04 Å) are nearly the same, and larger than oxygen (0.66 Å), whereas the electron distribution in all of these heterocycles is different. Therefore, the fact that selena-steroid (d) is active, whereas oxa-steroid (a) is inactive, suggests that steric, but not electronic factors are involved in the C-2, C-3 structural requirements of androgens. Confirmation for this was provided by the activities of telluriosteroid (e) which is somewhat more active than thiasteroid (b), and especially by disulfide (f), which is the most active compound in the series. The covalent radius of tellurium (1.37 Å) is considerably larger than that of sulfur. It is clear, therefore, that an exact isostere of a vinyl group is not mandatory for activity, but that a somewhat larger substitution may be made. Indeed, even the enlargement of the ring by the insertion of two sulfur atoms is permissible, as in (f). On the other hand, the 7-membered system A-homo-4-oxo-5α-androstan-17β-ol (i) is known to be inactive (69). These data indicate convincingly that an A-ring equivalent in size to a 6-membered or larger carbocyclic ring, and having atoms which flatten the ring in the vicinity of C-2 and C-3, or their replacment, is important for androgenic activity. Electronic characteristics of the A-ring are of minor importance. These findings were further supported by Liao et al. (70) who studied the specificities involved in the receptor binding and nuclear retention of various androgens and found that the bulkiness and flatness of the steroid molecule play a more important role in receptor binding than the detailed electronic structure at the Δ^4 bond of ring A.

In connection with the concept that only the steric, and not the electronic, characteristics of ring A are important in eliciting androgenic action, the synthesis of tricyclic compounds corresponding to steroids

lacking ring A, was described by Zanati and Wolff (11) who found that these compounds have significant androgenic activity. This finding is of importance in connection with the question of whether the intact steroid nucleus is a sine qua non for the production of various kinds of hormonal effects.

After having reviewed critically the several theories presented thus far on structure-activity relationships in steroid hormones, Vida (71) postulated that the steroid-receptor interaction is a three-dimentional attachment. The steroid acts like a porcupine with shorter and longer quills and only some of the quills really get attached to the receptor. The receptor is flexible in accommodating many of the structural features of steroids.

Unfortunately, all these hypotheses are based on in vivo biological data and, as recognized by these workers, assume that all of the compounds reach the receptor. In reality, however, androgenic activity is a net result of rates of absorption, tissue distribution, and metabolism. Moreover, as aptly pointed out by Wolff and Jen (67), lack of androgenic activity does not necessarily mean a lack of interaction with the receptor, for indeed many drugs exert their action by blocking the same receptors utilized by the corresponding protagonist compound.

In order to allow the assumption that its metabolism, to the extent that it occurs at all, will be by pathways different from normal steroids, and to assume that a given molecular modification need not have an effect on drug distribution (i.e., active or passive transport protein binding) common to both structural systems, a type of molecule, a 2-thia-A-nor steroid as described before, sufficiently different in structure from conventional androgens was introduced by Wolff et al. (72,73) as a probe of steroid-receptor interactions. By introducing activity-enhancing groups into 2-thia-A-nor steroid nucleus -- at the 7α, 10β, and 17α positions, Wolff et al. were able to demonstrated that the pharmacological effects of the modifications on

2-thia-A-nor steroids parallels their effects in the testosterone series. From this they concluded that these three modifications affect drug-receptor interactions, and not drug distribution or drug metabolism. The effects of some of these groups are through direct interaction with the receptor, in terms of the working hypothesis of the steroid-receptor complex proposed by Wolff et al. Thus, changes in activity due to modification of substituents at C-10 and C-17 are mediated through interaction of these groups with the receptor surfaces in contact with the β-face and α-face, respectively, of the steroid. On the other hand, the effect produced by the 7α-substituent is most probably due to a change produced in the conformation of the steroid itself, through conformational transmission (74). The axial 7α-substituent is involved in repulsive interaction with the 5α, 9α, and 14α positions. Therefore, the effect of this substituent would be to flatten the molecule toward the β-face. This flattening effect has been demonstrated by X-ray measurements in the case of the 9α-halogen compounds, in which the 9α-substituent interacts similarly with protons at 1α, 5α, 7α, and 14α positions (75).

(F) Uptake and selective retention of androgens by target organs.

Studies on the uptake and localization of androgens in various tissues were possible only after radioactive androgens became available. A study of the ability of target organs to specifically retain certain hormones is an essential step to understand the mechanism of action of steroid hormones and there are a number of reports dealing with the uptake and retention of radioactive androgens by the target organs, particularly by the rat ventral prostate.

In 1952 Barry et al. (76) were able to detect radioactivity in seminal vesicles after injection of radioactive testosterone of a low specific activity. Holmes (77) also demonstrated that the radioactivity was accumulated in the liver but not in other organs. Greer (78) injected ^{14}C-testosterone of somewhat higher specific activity and found a significant accumulation and retention of radioactivity in ventral prostate and seminal vesicles compared with muscle, adrenals, and salivary glands. It was found that the pattern of uptake did not follow that of blood. This indicates a selective retention of androgen by these target tissues. Pearlman and Pearlman (79) infused tritiated androstenedione (505 μg/hour/rat) into adult rats and found some degree of selective concentration of radioactivity in the ventral prostate, in which organ 15% of the ether-soluble unconjugated steroid fraction was in the form of 5α-androstan-3,17-dione. Harding and Samuels (80) also demonstrated that tissue radioactivity after injection of radioactive testosterone to the rats was slightly higher in the ventral prostate than in the plasma and other nontarget tissues such as liver and muscle. Both Pearlman's and Samuel's groups found that blood and liver contained a large amount of conjugated metabolites which are essentially absent in the ventral prostate. This suggests that the prostate has a selective process for the uptake of free metabolites but not conjugated androgens.

However, these early investigations on the fate of radioactive androgens of relatively low specific radioactivity can be discounted because of the unphysiologically high doses of androgens employed. With such very large doses of labeled androgens, the liver is swamped with the administered hormones, in which organ they are subject to a variety of transformations.

After administration of a low dose of highly radioactive testosterone to the castrated rats, the uptake and retention of androgens by male accessory organs was clearly demonstrated in several laboratories (81-85). In the case of ventral prostate and seminal vesicles, autoradiographic (86-88) and biochemical studies indicated that such selective uptake and prolonged retention is due to the binding of androgen to specific androphilic proteins both in the cytoplasm (83,89-91) and in the nuclei (81,83,84,92,93). The uptake of androgens in vivo also has been demonstrated in the ductus deferens and the epididymis of the rat (94). Similar uptake patterns also have been found after the injection of ^{3}H-5α-dihydrotestosterone (95) and ^{3}H-androstenedione (96). The accumulation of testosterone in the hypothalamus, cerebrum and pituitary of the guinea pig after an injection of radioactive testosterone was described by Resko et al. (97).

The uptake and retention of testosterone and 5α-dihydrotestosterone by rat ventral prostate in vitro was demonstrated by Hansson (98). It was found that the radioactivity in the prostate was about four times higher than in the muscle after incubation of slices of ventral prostate and skeletal muscle from castrated rats with ^{3}H-testosterone (9.7×10^{-10}M). The addition of non-radioactive testosterone (2 μg/ml) to the incubation medium reduced the prostatic retention of androgen almost to that in the muscle specimens. Incubation with ^{3}H-5α-dihydrotestosterone (8×10^{-10}M) gives essentially the same results as incubation with ^{3}H-testosterone (98). Some evidence indicating a selective uptake of androgen by the prostate and seminal vesicles of

infantile rats *in vitro*, has been reported (99). The selective retention of 5α-dihydrotestosterone by prostate cell nuclei *in vivo* can be reproduced *in vitro* by incubating minced prostate glands with radioactive testosterone, androstenedione, or 5α-dihydrotestosterone (81,83,84). Evidence available (81,83) strongly indicates that 5α-dihydrotestosterone is not retained by the outer nuclear membrane, but rather inside the nuclei and appears to be tightly bound to nuclear chromatin.

The conversion of testosterone to 5α-dihydrotestosterone and other unconjugated steroids by prostatic tissue *in vitro* has been exhaustively reviewed (100). Since 1968, many publications have suggested that 5α-dihydrotestosterone might be an active form of testosterone in some androgen-responsive organs and appears to associate selectively with nuclear components of these organs. Bruchovsky and Wilson (92) found that after injection of ^{3}H-testosterone into normal or functionally hepatectomized rats, radioactive testosterone, 5α-androstan-3α,17β-diol, androsterone, and 5α-dihydrotestosterone were detectable in the cytoplasm of the ventral prostate within 1 min, whereas only 5α-dihydrotestosterone, and small amounts of testosterone as such, were recovered from prostatic cell nuclei for as long as 2 hours after injection of the hormone. Significant amounts of free 5α-dihydrotestosterone were found only in the prostate, seminal vesicles, and preputial gland. Small amounts of 5α-dihydrotestosterone were detectable in kidney and plasma, while virtually none was recovered from liver, heart, gut, and lungs (92). Furthermore, it has been shown (81,92) that in the presence of a $NADPH_2$-generating system prostatic nuclei convert testosterone to 5α-dihydrotestosterone, whereas prostatic cytoplasm, in addition, reduces 5α-dihydrotestosterone to androstandiol. It was concluded that testosterone is reduced in the nuclei of androgen-sensitive tissues to 5α-dihydrotestosterone, which might function as an "active form" of the hormone.

Anderson and Liao (81) independently demonstrated that 5α-DHT represents the major (>75%) radioactive product associated with cell nuclei isolated from the ventral prostate when 7α-^{3}H-testosterone is injected into rats. Testosterone was a minor (5-25%) component; no other radioactive metabolites of testosterone were found in the nuclei. Less than 5% of the cytoplasmic radioactivity was in the form of free 5α-DHT. Anderson and Liao also demonstrated that the formation of 5α-DHT from radioactive testosterone by rat prostatic nuclei in vivo could be reproduced in vitro by incubation of minces of prostate gland with ^{3}H-testosterone. When cell nuclei were isolated from prostatic cells at the end of the incubation in vivo, only 5α-DHT (75-90%) and a small amount of testosterone could be identified. Such a selective retention of radioactivity (largely as 5α-DHT) by nuclei of minced prostate in vitro was not observed with thymus, brain, diaphragm, and liver (81). According to Fang and Liao (101), 5α-DHT formed from testosterone was retained by the rat ventral prostate for at least 6 hours, and long after virtually all radioactive steroids had disappeared from both blood and a number of tissues which are relatively insensitive to androgens. Tveter and Aakvaag (102) also found that 1 hour after administration of ^{3}H-testosterone, unchanged hormone represented only 3-17% of the total radioactivity in accessory sex organs, whereas 5α-DHT accounted for as much as 70% of the radioactivity in prostate and seminal vesicles.

Bruchovsky and Wilson (92) and Anderson and Liao (81) discovered a chromatin-bound steroid 5α-reductase in prostatic cell nuclei that catalyzes a reduction by NADPH of the double bond in ring A of testosterone to yield 5α-DHT. Later, Frederiksen and Wilson (103) delineated many properties of the 5α-steroid reductase in rat ventral prostate cell nuclei. Earlier experiments by Shimazaki et al. (104,105) indicated that microsomes from rat ventral prostate also contain a similar C_{19}-steroid 5α-reductase.

The mechanism of androgen uptake and concentration by rat ventral prostate in organ culture has been studied (106). It was found that 5α-DHT was the principal intracellular androgen whether the hormone in the incubation medium was testosterone or 5α-DHT, and intracellular to extracellular gradients for 5α-DHT were greater than those for testosterone under all conditions studied. These findings are compatible with the possibility that the conversion of testosterone to 5α-DHT and subsequent binding of 5α-DHT to receptor sites within the tissue serve to keep the activity of intracellular testosterone low and to promote passive diffusion down an activity gradient from blood. The net effect of such a system is the development in the prostate of a higher concentration of total androgen (testosterone plus 5α-DHT) than in the medium.

It is well known that 5α-DHT exhibits an even greater potency than testosterone itself in a number of androgenic bioassay systems (84). This fact, together with the rapid formation of large amounts of 5α-DHT by androgen-responsive tissues and its retention in their nuclei, strongly hints that 5α-DHT may be an active form of testosterone in at least some androgen-sensitive cells. However, various investigators have demonstrated that systemic 5α-DHT, in the free or propionate form, is relatively ineffective in comparison with the analogous form of testosterone in eliciting sexual behavior in male rats (107-111). From these, it was concluded that testosterone probably does not activate male sexual behavior via conversion to 5α-DHT (111). Furthermore, Liao et al. (70) recently found that many potent synthetic androgens such as 7α,17α-dimethyl-19-nortestosterone could bind directly to cytosol receptor and to cell nuclei of ventral prostate without a metabolic conversion by prostate Δ^4-3-ketosteroid-5α-oxidoreductase that seems to be required for the action of testosterone in prostate.

(G) Androgen receptors

In an incisive essay, Hechter and Halkerston (112) point out that the basic problem of the mechanism of hormone action at a molecular level is to account for the chemical nature of the "primary receptors" for any given hormone in responsive cells as well as the physico-chemical details of interaction of the hormone with these receptor substances. It is notoriously hard to construct theories of hormone action which are not predicated on the existence of such primary receptors. Often it is assumed that only one or, at most, relative few types of such receptors are present on the surface or within susceptible cells, that these entities are macromolecules of one form or another, and that attachment of the hormone to these receptors occurs via various noncovalent forces.

An approach to the problem of sex hormone receptors is to examine the nature and binding to specific cellular constituents of the radioisotope derived from labeled hormones of very high specific radioactivity, either after injection of the labeled hormone or its incubation with isolated tissue preparations. The pioneer studies of Jensen and his collaborators (113,114) in which labeled steroidal estrogens and synthetic compounds such as hexestrol were used showed that the uterus and a number of other estrogen-responsive tissues contain proteids with a remarkable and specific avidity for estradiol-17β and some synthetic estrogens. Despite extensive metabolism of estradiol in the animal giving rise to a variety of metabolites in the blood, only unchanged estradiol was taken up by the uterus of the immature rat (113) or mouse (115). These "estrogen receptor" proteids appear to be responsible for the selective uptake and retention of estrogens by organs such as the uterus and vagina. The investigation of androgen receptor, however, is complicated by the ability of target tissues to convert testosterone to 5α-dihydrotestosterone and to retain this active metabolite in preference to

testosterone itself. A number of investigators have studied the tissue specificity of the nuclear binding, the characteristics of the binding component and the intranuclear binding site.

Bruchovsky and Wilson (93) studied the intranuclear binding of radioactive hormone in prostate after the intravenous administration of ^{3}H-testosterone to rats. Nuclei obtained by sucrose density gradient centrifugation were extracted with buffer containing 0.6 M NaCl. By gel filtration of nuclear extracts on Sephadex G-200 and by the use of digestive enzymes, such as DNase, RNase, and Pronase, it was shown that 5α-DHT was bound to an acidic nuclear protein. In addition, several properties of this binding phenomenon were characterized; the binding was stable to freezing for as long as 8 days, stable to short term incubation at 20° but not at 37°, and partially stable to repeated gel filtration on Sephadex. A salt-extractable androgen receptor of rat prostatic nuclei (molecular weight 100,000-120,000) was also shown by Mainwaring (116).

These results were extended by Fang, Anderson, and Liao (83). It was found that the DHT-bound protein extracted from the labeled nuclei by a buffered salt solution migrated in a sucrose gradient centrifugation with a sedimentation constant of 3.0 ± 0.3 S. The cytosol fraction of rat ventral prostate homogenates also contained a specific DHT-binding protein which has a sedimentation constant of 3.5 ± 0.3 S. The affinity of the binding reaction was high; their results are consistent with $K_d \sim 10^{-10}$M. The selective retention of DHT by cell nuclei of ventral prostate could be shown by incubating minced prostate with ^{3}H-testosterone, ^{3}H-DHT, or ^{3}H-4-androsten-3,17-dione *in vitro*. The cytosol 3.5 S protein bind DHT spontaneously at 0°. At 37°, the DHT-protein complex extracted from prostate cell nuclei gradually releases the bound DHT. On the other hand, incubation of the cytosol protein at temperatures between 15-50° enhanced the DHT-binding capacity. Heating of

the cytosol protein at temperature above 50° for 10 minutes destroyed such binding ability. It was found (83) that if ^{3}H-DHT was incubated with isolated prostate cell nuclei, then extracted with a 0.4 M KCl solution and analyzed by gradient centrifugation, there was no formation of the 3 S DHT-protein complex. Salt extract of nuclei not previously exposed to ^{3}H-DHT also did not form the 3 S complex when ^{3}H-DHT was supplemented to the extract. However, when the cytosol fraction not sedimented at 100,000 g for 1 hour was added to the isolated nuclear fraction and exposed to ^{3}H-DHT, the 3 S DHT-protein complex could be extracted from the reisolated nuclei. Their finding suggested that prostate cell nuclei have specific sites that can retain a specific DHT-protein complex but not the protein moiety or DHT alone. However, Unhjem (117) has shown that cytosol factors are not critical. Rennie and Bruchovsky (118) also confirmed the latter observation.

Fang and Liao (119) found that the 3.5 S cytosol fraction could be separated into two subfractions, complex I (α-protein-DHT) and complex II (β-protein-DHT), by an ammonium sulfate fractionation and a Sephadex gel filtration. At 0° and pH 7 ^{3}H-DHT, associated with the cytosol 3.5 S protein (complex I), could be exchanged with nonradioactive DHT whereas ^{3}H-DHT, bound to the nuclear receptor protein, did not show any significant exchange with the nonradioactive DHT within several hours. The complex II had many properties (fractionation patterns, heat stability, and others) similar to that of 3 S nuclear DHT-receptor protein complex. In cell-free systems, complex II but not complex I bound firmly to cell nuclei isolated from prostate but not to liver nuclei. Complex II may be the receptor-DHT complex (or its immediate precursor) retained by prostate cell nuclei <u>in vivo</u>. Although there is no direct evidence that the 3.5 S proteins (or in complex with DHT) are the precursors of the receptor protein which retains DHT in the prostate cell nuclei, some factors may be present in the ventral prostate for such trans-

formation. Cell nuclei of rat ventral prostate appear to contain specific nuclear acceptors which can selectively retain complex II. The nuclear acceptor was heat sensitive and rapidly destroyed when nuclei were incubated alone for 10 minutes at a temperatures higher than 50°. Complex II is even less stable to heating. At 37° about one-half of the bound DHT is released from complex II in 10 min. Complex II, by itself, is not retained firmly by prostate cell nuclei in the absence of DHT. This suggests that binds to the receptor protein in such a way that the complex formed will have the structural requirement to fit the binding site of the prostate nuclear acceptor. Other steroids such as 5β-DHT, testosterone, 5α-androstan-3,17-dione, 3α (or 3β),17β-dihydroxy-5α-androstane, 4-androsten-3,17-dione, 17β-estradiol, cortisol, and progesterone are not effective for this purpose. As reported elsewhere (84), a DHT-protein complex extracted from microsomes of minced prostate previously incubated with ^{3}H-DHT also sedimented at a rate similar to that of nuclear DHT-protein complex.

Other workers have independently confirmed the existence of rat prostatic and nuclear high-affinity receptors (HAR) for androgens but disagreed with the estimates of Fang et al. about the sedimentation coefficient of the cytosol receptor. Mainwaring (91,120), Unhjem, Tveter, and Aakvaag (90), Baulieu and Jung (121), and Mainwaring and Peterkin (122) found that the cytosol HAR-bound androgen sedimented at ~8-9 S (molecular weight ~280,000); the molecular weight is therefore approximately double that of the nuclear complex. The 8 S receptor preferentially binds DHT; binding is inhibited by cyproterone acetate. The receptor is reversibly salt dissociable into a 4 S form and co-exists with a less specific androgen-binding protein. Estimates of the sedimentation coefficient of the latter vary between 3.5 and 4.5 S. The integrity of sulfhydryl groups is necessary for the binding reaction (117).

Liao et al. (123) found that complex II (and not complex I) formed aggregates easily upon standing at 0° to 2° and the aggregates could be dissociated to the 3 S complex in the presence of 0.4 M KCl. It was suggested that the 8-9 S complex could be an aggregated form of complex II through an intra- or intermolecular association. Baulieu and Robel (124) found that if rats had been castrated for 3 days or more the ratio (8 S-HAR : 4 S-binding protein) decreased. This, they suggest, may account in part for the discrepancy between their findings and those of Liao et al. and may indicate that the receptor synthesis is androgen-dependent. However, Sullivan and Strott (125) presented evidence for an androgen-independent mechanism regulating the levels of receptor in target tissue. When the amount of radioactive DHT bound to high molecular weight molecules in the 8-10 S fraction of the 100,000 g supernatant was measured at intervals following castration, the receptor concentration (and activity per mg of cytosol protein or per μg of DNA) declined steadily to immeasurable levels by the 4th day following castration. However, whereas the size of the prostate and the DNA content per prostate and per mg of tissue continued to fall, the receptor concentration per mg of tissue and μg of DNA increased to levels similar to those found 1 day following castration. The maximum was reached by the 8th day after castration and was maintained for at least 6 weeks. The same phenomenon was observed in animals which were either hypophysectomized or adrenalectomized at the time of castration. Thus, restoration of receptor is apparently not mediated by adrenal androgens or pituitary hormones. Based on this, they concluded that there is an androgen-independent mechanism for providing receptor to receive the androgenic stimulus for growth and development as well as for maintenance of the mature prostate.

The binding of the prostatic HAR-DHT complex to chromatin in prostatic nuclei has been shown by both Mainwaring and Peterkin (122) and Steggles et

al. (126). This process is both steroid and tissue specific, though prostatic nuclei can also take up the HAR-DHT complex from another target tissue -- seminal vesicles (122). Certain aspect of the specificity of cytoplasmic binding and of nuclear uptake have been confirmed by Tveter (127) and by Parsons, Mangan, and Neal (128) but perhaps the strongest evidence for the physiological role of the androgen HAR comes from the work of Gehring, Tompkins and Ohno (129) who showed that in the kidneys of androgen-insensitive mutant mice there was a decrease in the amounts of cytoplasmic HAR and in the quantity of DHT taken up by the nucleus. Their results also indicate that there are two different classes of cytoplasmic DHT receptors, thus adding additional weight to the observation of Fang and Liao (119).

A very recent report by Kasuya and Wolff (130) described the binding of the three radioactive androgens DHT, 17α-methyl-5α-androstan-17β-ol, and 17α-methyl-5α-androst-2-en-17β-ol to minced rabbit ventral prostate and concluded that there are three different binding sites for such radioactive androgens: the "classical" DHT site, as well as separate sites for the olefin and hydrocarbon derivatives. Whether these are receptors in the cytosol or nucleus, or both, and whether they are different sites on the same macromolecule or represent different macromolecules is, of course, still an open question.

Tveter et al. (131) found that when human prostatic tissue (normal prostate, benign prostatic hyperplasia and prostatic cancer) was incubated with ^{3}H-testosterone or ^{3}H-DHT, results almost identical to those for rat prostate were obtained. The human prostate can selectively uptake and retain androgens for a prolonged period of time.

Androgen receptors also have been demonstrated in cytosol from rat seminal vesicles (85,132). Uptake of testosterone is inhibited competitively by cyproterone (132); calculation made from these data indicates that the

affinity for testosterone is high (K_d ~4 x 10^{-9} M). Presumably the affinity for DHT is even higher. Liao et al. (123) were also able to detect 7 S complex in the 0.1 M KCl extract of the rat seminal vesicle. By increasing the salt concentration gradually to 0.4 M KCl, 5α-DHT binding complexes having sedimentation constants between 3 and 6 S have been observed for the seminal vesicle systems. It appears that the receptor protein(s) can exist in several polymerized forms or in association with other cellular components. However, it is not clear which of these forms are actually present in the intact cells or involved in the action of 5α-DHT.

Besides ventral prostate and seminal vesicles, rat epididymis has also been found to contain androgen-binding protein. The mammalian epididymis, which is known to be an androgen-dependent organ, is uniquely located to receive this hormone both from the blood stream and directly from the testis by way of fluid flowing into the epididymis through the efferent ducts. Blaquier et al. (133,134) have demonstrated that rat epididymis is a target organ for androgens and showed that the epididymis possessed the ability to selectively take up androgens which concentrate intranuclearly, paralleled with a marked increase in nucleic acid metabolism (135). Furthermore, evidence for the presence of a specific cytoplasmic receptor for androgens has been presented by Blaquier (136) and Ritzen et al. (137). The cytoplasmic receptor for androgens in rat epididymis has a sedimentation constant of 4 S. The complex has a dissociation constant of between 10^{-9} and 10^{-10} and binding is stabilized by 2-mercaptoethanol. The existence of intranuclear receptor for androgens was later reported by Blaquier (138). Several characteristics of this receptor, such as: sedimentation coefficient, thermolability, protein composition and resistance to DNase, are similar to those described for nuclear receptors for androgens in prostate (83,139) and for estrogens in uterus (114). Moreover, the hormone seems to be transferred from the

cytoplasm to the nucleus bound to the receptor. This finding is significant in view of the recent demonstration that sperm maturation is dependent upon the presence of androgens (140,141).

A high-affinity androgen-binding protein in rat testes was reported by French and Ritzen (142) who demonstrated that the androgen-binding protein is formed in the rat testis and transported to epididymis via the efferent duct fluid. It was suggested that as testicular fluid passes through the caput of the epididymis, a major protein of the androgen-binding protein either loses its binding activity or disappears from the soluble supernatant because of absoption from the lumen and uptake by an insoluble subcellular organelle of epididymal cells. Androgen binding protein in the testis cytosol fraction was found to be identical to an androgen binding protein in rat epididymis (143,144).

Testosterone cytosol receptor was also found (145) in the rat levator ani muscle, which is very similar to the prostatic receptor, but binds more testosterone than DHT. It was interesting that 1 nM ^{3}H-DHT gave a much smaller radioactive 8-10 S peak than 1 nM ^{3}H-testosterone as this was the reverse of what had been described for the prostate (121). Competition experiments verified that the same binding sites were involved with both steroids and confirmed the greater affinity for testosterone (145). These observations demonstrate the presence of a steroid-binding protein in a muscle and favor a direct action of testosterone on the levator ani as do the effects obtained <u>in vivo</u> (146). This is well in line with the finding that the ratio of the growth promoting effect on ventral prostate and levator ani of testosterone was greater than that of DHT. It was found (145) that the K_a for testosterone of the levator ani muscle cytosol receptor (approximately $5x10^{8}$ M^{-1}) was somewhat less than the K_a for DHT of prostatic receptor, and that the number of receptor sites per mg of cytosol protein was

smaller in muscle than in vetral prostate.

In 1973 Giannopoulos (147) described studies on the *in vivo* and *in vitro* uptake and retention of testosterone by the immature rat uterus and on the interaction of testosterone with nuclear components. His results demonstrate that the immature rat uterus contains cytoplasmic and nuclear binding components (receptors) with high affinity and specificity for testosterone. Thus the uterotrophic and anti-uterotrophic action of testosterone appears to be a direct action of the hormone by a mechanism distinct from that of estradiol. This data also demonstrate that the uterine androgen-binding components are different from those found in the rat prostate with regard to their relative affinity for testosterone and DHT. Therefore, the intracellular active form of androgen may vary from tissue to tissue. Whereas DHT appears to be the major active androgen in the rat prostate, in other tissue such as the uterus the predominant active androgen may be testosterone itself since both *in vivo* and *in vitro* studies showed that the immature rat uterus lacks the capacity to convert testosterone to DHT or estrogens, to any significant extent.

The formation and binding to nuclear proteids of DHT in many androgen-sensitive organs appears to be related to the mechanism of action of anti-androgenic substances which includes the compounds cyproterone acetate and cyproterone. These synthetic compounds strongly inhibited the uptake of radioactive androgens by ventral prostate and seminal vesicles *in vivo* (83, 101,132,148-151). In the case of ventral prostate, this was accompanied by a decrease in the retention of DHT by cell nuclei (83,101,151). In the *in vitro* tissue immersion experiments, cyproterone and its acetate at concentration as low as 1 μM significantly inhibited the formation of a specific DHT-protein complex in the prostatic cell nuclei (83). Since ^{3}H-DHT rather than ^{3}H-testosterone was used in this experiment, the inhibition was not at the stage of the enzymatic conversion of testosterone to DHT. Fang and Liao

(119) also showed that cyproterone acetate added to the cell-free system did inhibit the retention of 3 S DHT-protein complex by prostatic cell nuclei. Similar inhibition of testosterone uptake by accessory reproductive glands by antiandrogen 17α-methyl-B-nortestosterone, as well as a lack of effort of estrogens *in vivo*, has been reported (102). Estradiol-17β, diethylstilbestrol, and progesterone, but not cortisol, also suppressed the retention of DHT by prostate cell nuclei *in vitro*, but to a much lesser extent than cyproterone acetate (83). The antagonistic action of antiandrogens strongly suggests the contention that the binding of DHT to the receptor proteins is germane to the stimulation of prostate growth and function by androgens.

Currently, it is believed that in the target tissues steroid hormones are bound by cytoplasmic receptors of high affinity and specificity; that the steroid-receptor complex moves to the nucleus where physiological changes are initiated via a modification of genetic expression. This was described by Jensen (114) as occuring in the "two-step" temperature-dependent process.

(H) Dissociation of androgenic and anabolic receptors.

A controversy has raged for years as to whether the growth of the levator ani muscle of the castrated rat is a valuable index for measuring the myotrophic activity of steroidal hormones or merely a response of part of the male reproductive system to androgenic stimulation (28-30). Nevertheless, this is the only test in general use for rapid pharmacological screening and there is a definite correlation between the myotrophic properties of certain steroids and their ability to induce nitrogen retention -- the true anabolicity (26). Moreover, there is something unique about the response of this muscle to certain steroids in that it may be maximally stimulated by some compounds which have only weakly androgenic effects on the ventral prostate and seminal vesicles. On the other hand, most androgens also promote nitrogen retention and stimulate the levator ani. Thus, the question of a true distinction between androgenic and anabolic properties has remained unresolved.

In 1971 Steinetz et al. (152) reported the dissociation of anabolic and androgenic properties of steroids by the use of specific inhibitors, namely A-norprogesterone (ANP), a pure antiandrogen with no other known effects (153), and dexamethasone (Dex), an anti-anabolic glucocorticoid with no other demonstrable androgenic or anti-androgenic activity in the rat. The compounds to be compared for their effects on the levator ani (LA) were testosterone propionate (TP), a potent androgen with protein anabolic properties, and methandrostenolone (M), an anabolic agent with weak androgenic properties. TP and M were given in doses which produced equivalent increases in LA weight. It was found that ANP completely antagonized the effect of TP but not that of M on LA weight. ANP inhibited both TP- and M-induced growth of seminal vesicles (SV) and ventral prostate (VP). Dex markedly reduced the response of the LA to both TP and M, while not influencing their effects on SV and VP.

Neither Dex nor ANP alone or in combination influenced organ weights. These results led to postulate that the LA muscle may have two distinct sets of receptors: one for anabolic and one for androgenic activity. Although stimulation of either type of receptor leads to muscle growth, they may be distinguished by the use of specific inhibitors. The SV and VP seem only to have androgenic receptors, as their stimulation by either M or TP is unaffected by Dex but inhibited by ANP. The data also supported the view that M stimulates primarily anabolic receptors of the La muscle, while TP stimulates both types of receptors. However, it appears to be more sensitive to blockade by the antiandrogen, ANP, than by Dex. These hypotheses were further supported by the finding that the LA of hypophysectomized rats responded to growth hormone as well as to TP, whereas the SV responded only to TP (154).

(I) Mechanism of action of steroid hormones in inducing remission of breast cancer.

The precise mechanism of action of the sex hormones in inducing remission of mammary cancer has not been determined. The most likely mechanism is the direct (local) effect on the tumor and its stroma. The inhibitory effect of androgens on mammary carcinoma in rats seems to involve increase in activity in the normal tissues at the expense of the malignant cells, as reflected in the activity of specific enzymes in the tissue (155). The local application of testosterone to one mammary gland of a castrated rat will induce growth stimulation of only the mammary gland under observation and not of the others (156). It has not been established whether the effect of androgen in causing regression of breast cancer is primarily on the epithelial elements or on the tumor stroma.

In regard to a local tissue effect, the concept of receptor proteins (83,113,114,119) in the tissues which bind the hormone provides a feasible hypothesis for the explanation of the remissions produced by administration of therapeutic agents. Such a theory would presuppose that the administered compound prevents binding by the tumor of the appropriate sex hormone thus preventing the sex hormone from exerting a biological effect. There are numerous examples of these interaction at the in vitro level (157). It also has been suggested that some breast cancers can take up higher concentration of the ^{3}H-estrogens in vivo and in vitro (158,159). Thus, if the hormone dependency or estrogen responsiveness of a breast cancer can be indicated by the degree of uptake of estradiol in the tissue, it might be a reasonable approach to measure the amount of the receptor (estrogen-binding capacity) in the tissue, for establishing a basis for the selection of suitable patients for endocrine therapy. In the study on the measurement of estrogen-binding capacity in the hormone-dependent and hormone-independent rat mammary

tumors, Nomura et al. (160) found higher estradiol-binding capacities in the cytosols of tumors responding better to oophorectomy, and lower capacities in more autonomous tumors. These findings are consistent with those reported by others by different methods (159). It also has been found (160) that in the hormone-dependent tumor cytosol, very low concentration of unlabeled estradiol competed with ^{3}H-estradiol for the binding sites, showing the binding to be highly specific. On the other hand, unlabeled estradiol showed little competition with ^{3}H-estradiol for the autonomous tumor cytosols.

A significant amounts of estradiol receptor has been found in a stable human cell line (MCF-7) derived by pleural effusion from a breast cancer patient (161). This binding protein is specific for estradiol and has a K_d equal to 2.5 nM, a sedimentation constant of 4.0 S (and 9.2 S), and a mechanism capable of transporting the estradiol into the nucleus.

Recently, McGuire et al. (162) concluded that binding of estrogen in vivo and in vitro depends on the presence of a cytoplasmic receptor protein that acts in the nucleus after binding estrogen molecules. This estrogen receptor is present in both rat and human dependent mammary tumors, but is usually missing in autonomous mammary tumors. The presence or absence of estrogen receptor may thus be an indicator of the retention or partial loss of the complete endocrine regulatory unit, which mediates the interacting effects of many hormones including prolactin and estrogen on the normal mammary gland.

Although remissions of cancer may be produced by hormone therapy or ablation of endocrine glands, such remissions are rarely permanent. Very much more remains to be done concerning the biochemical changes associated with these remissions and particularly why these so often last for only short periods.

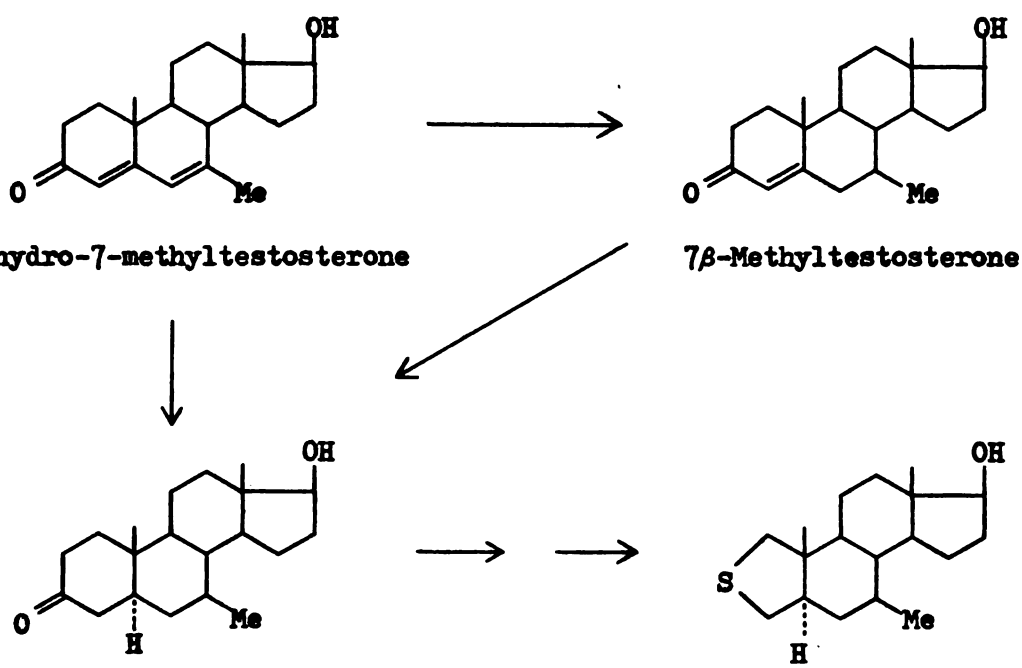
PART III

CHEMISTRY

(A) Discussion

Only a few androstane derivatives containing the 7β-methyl group, as shown in Table 1, have been studied and tested for androgenic-anabolic activity. However, only preparations of 7β-methyltestosterone (163) and the corresponding 17α-methyl derivative (48) have been reported. Neither synthesis nor physical properties of the rest compounds shown in Table 1 was described.

Since 7β-methyl-5α-dihydrotestosterone and its 2-thia-A-nor analog were sought in the present work, 6-dehydro-7-methyltestosterone was chosen as a key intermediate, and its preparation was investigated. Upon catalytic hydrogenation, 6-dehydro-7-methyltestosterone would give 7β-methyl-5α-dihydrotestosterone which would ultimately afford the corresponding 2-thia-A-nor analog, 7β-methyl-2-thia-A-nor-5α-androstan-17β-ol.



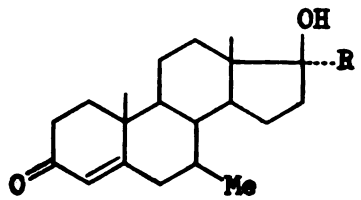
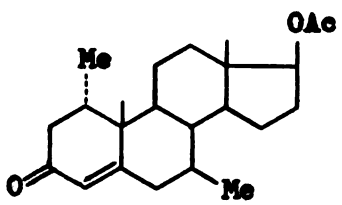
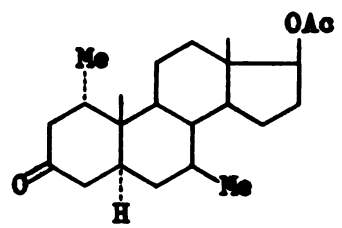
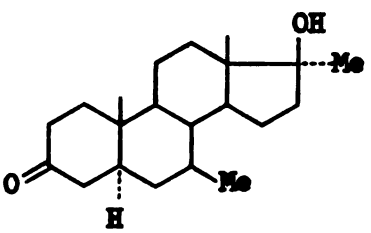
6-Dehydro-7-methyltestosterone

7β-Methyltestosterone

7β-Methyl-5α-dihydrotestosterone

7β-Methyl-2-thia-A-nor-5α-androstan-17β-ol

Table I. 7β-Methyl substituted androstane derivatives

Structure	Name	Ref.
	R = H, 7β-Methyltestosterone	70,163
	R = Me, 7β,17α-Dimethyltestosterone	48,50,70
	1α,7β-Dimethyltestosterone 17-acetate	164
	1α,7β-Dimethyl-5α-dihydrotestosterone 17-acetate	164
	7β,17α-Dimethyl-5α-dihydrotestosterone	70

There are three synthetic methods for the preparation of this intermediate appeared in the literature (48,163). The first, as shown in Fig. 3, was 1,6-addition of Grignard reagent to 6-dehydro-17α-methyltestosterone (see Ref. 165 for discussion of 1,6-addition of Grignard reagents). This is the shortest route to obtain a mixture of 7α- and 7β-methyl steroids in one step (48). In the same way, 7β-methyltestosterone was also prepared from 6-dehydrotestosterone (166). According to Campbell and Babcock (48), the mixture of 7α and 7β-methyl epimers obtained by this method was not separated readily by chromatography on Florisil or by paper chromatographic techniques. The separation was achieved by treatment of the mixture with chloranil, in which the 7β-methyl epimer was dehydrogenated to afford 6-dehydro-7,17α-dimethyltestosterone. Chromatography then readily separated the product from the unchanged 7α-methyl epimer. This method yielded only a small amounts of the 7β-methyl steroid and the major product was the 7α-methyl epimer. For this reason this scheme was not adopted.

The second route (shown in Fig. 4), which was also utilized by Campbell and Babcock (48) as shown in Fig. 3, employed the readily available androst-5-en-3β,17β-diol diacetate (I) which was converted to the 7-keto steroid (II) by allylic oxidation at C-7 position with t-butyl chromate in acetic acid and acetic anhydride solution (167). The nature of the reagent used is not clear. It may actually be diacetyl chromate formed by a reaction of the t-butyl chromate with the acetic anhydride which is added to the solution (168). Since the oxidation is acid catalyzed, it may involve mixed anhydrides, or perhaps the cation $(HCrO_3)^+$ in which the electron-accepting property of the O=Cr bond is enhanced by the positive charge (169). As shown below, the initial reaction could be the formation of epoxide or more probably some polar intermediate easily converted into a derivative of Cr^{IV}, which would undergo tautomerization and hydrolyze at the O-Cr bond to give,

Fig. 3. Preparation of 7-methyl substituted testosterone

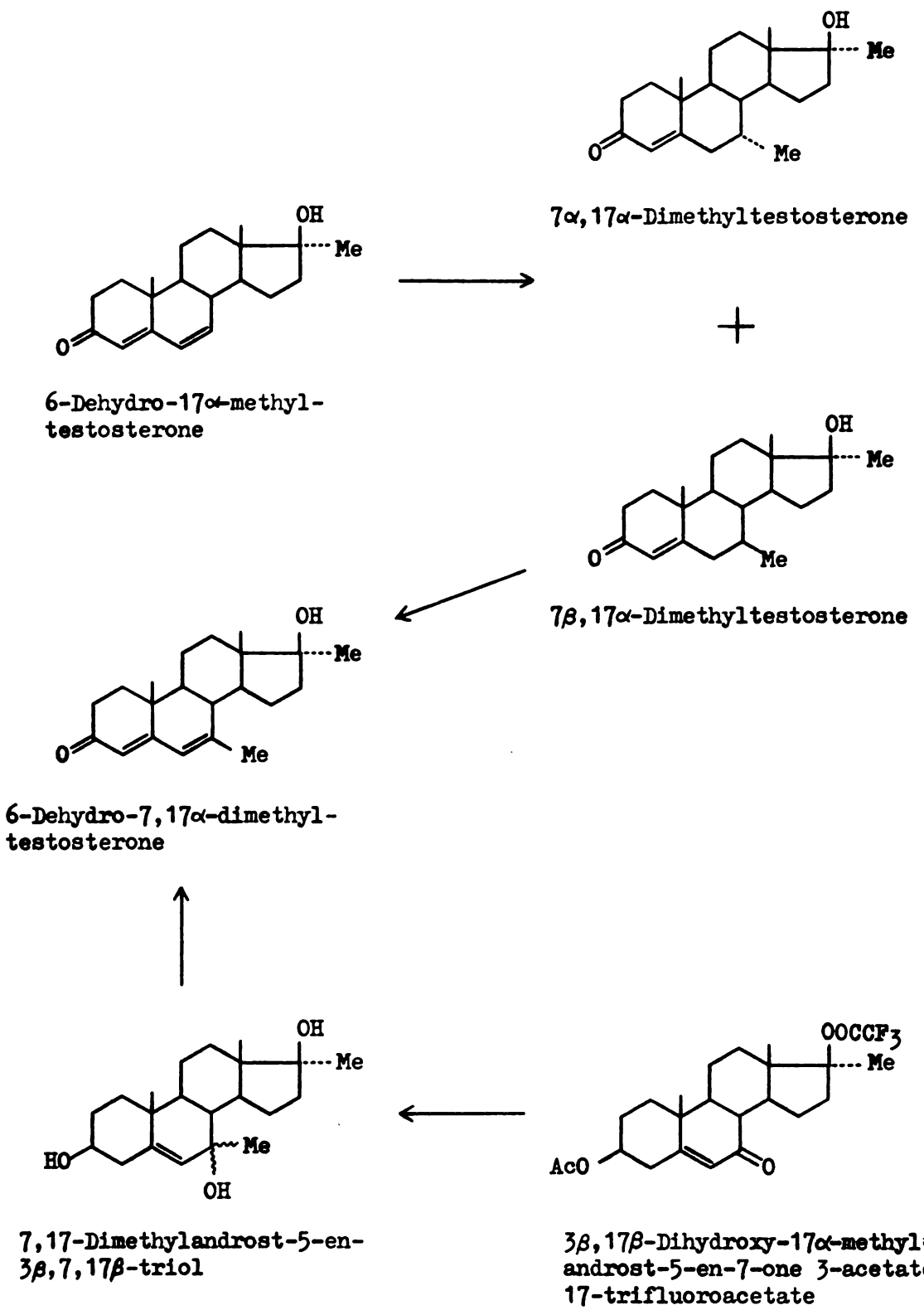
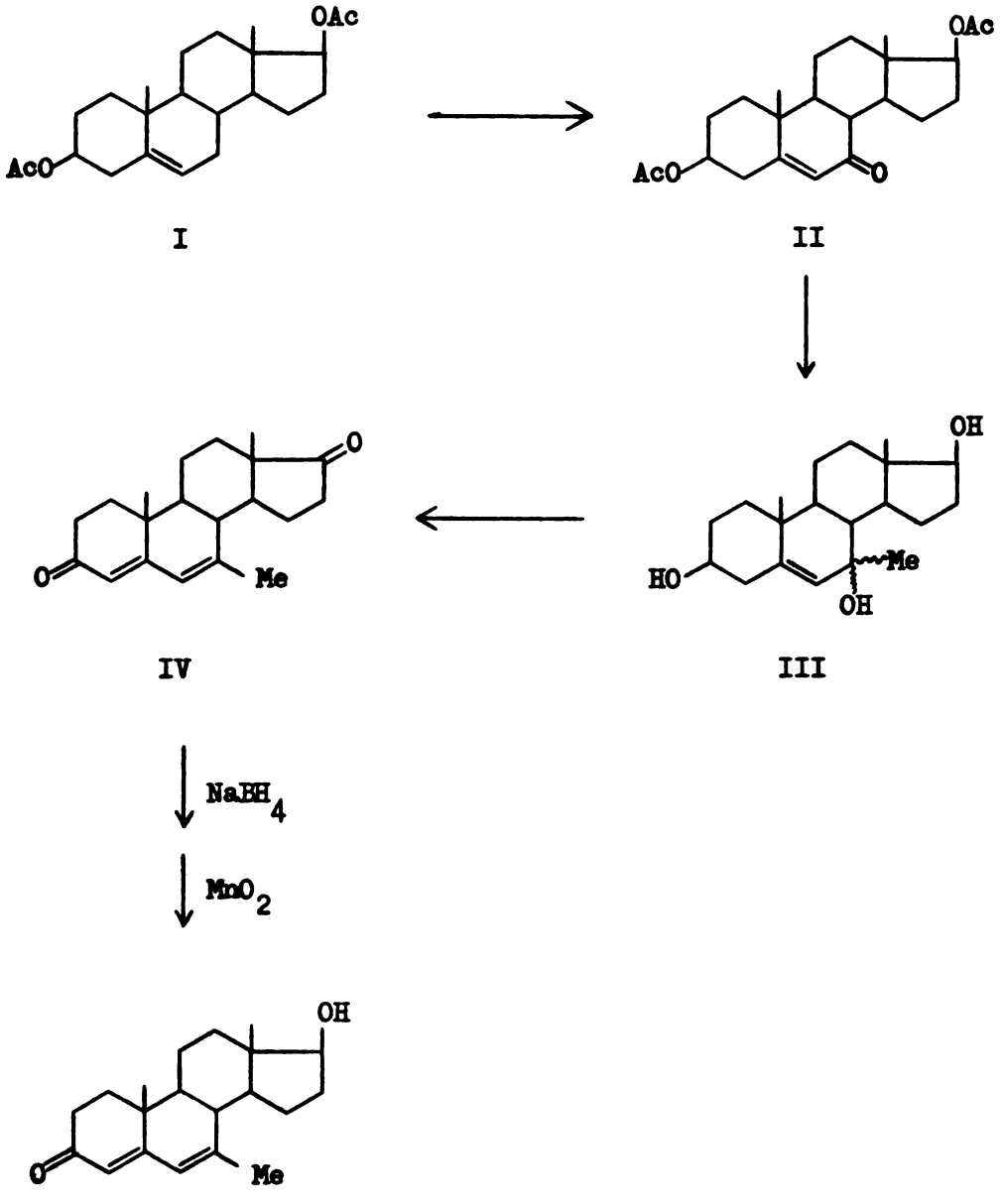
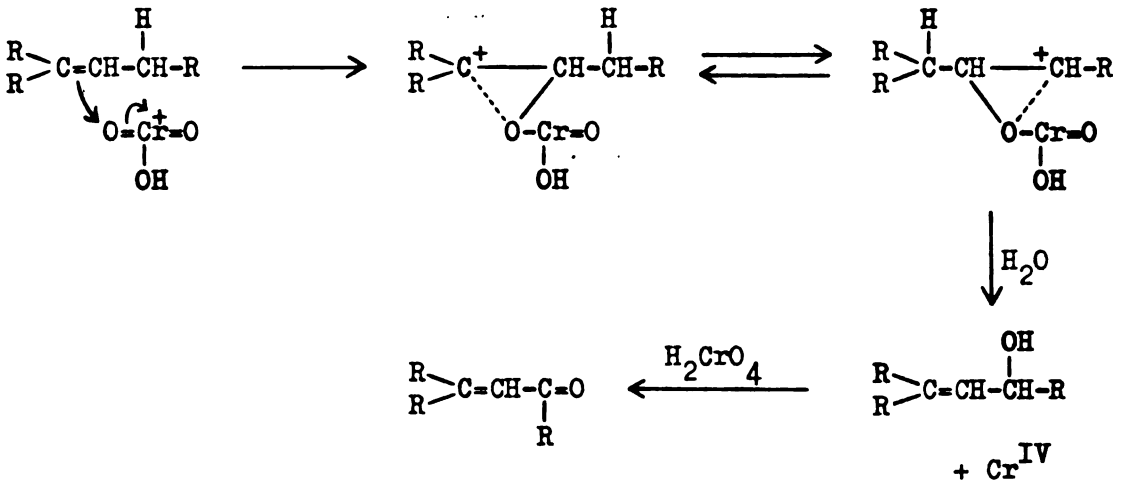


Fig. 4. Preparation of 6-dehydro-7-methyltestosterone

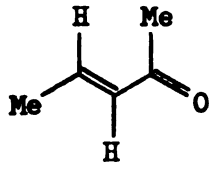


6-Dehydro-7-methyl-testosterone

after further oxidation, the α,β-unsaturated ketone. As described later, it was found that the oxidation of Δ^5 steroid occurred on the allylic center of C-7 rather than of C-4 (170).

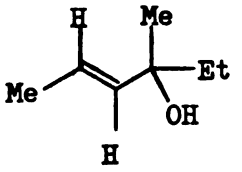


When treated with methyllithium or methylmagnesium bromide, the 7-keto steroid (II) afforded the 7-methylcarbinol (III), which may have been a solvate or a mixture of epimers at C-7, since it resisted purification. In the reaction of Grignard reagents with α,β-unsaturated aldehydes and ketones, 1,4-addition can take place in competition with normal 1,2-addition. The balance between 1,2- and 1,4-addition is often controlled by steric factors. In general, substitution at the carbonyl group increases 1,4-addition, while substitution at the double bond increases 1,2-addition. In most cases both products are obtained, but α,β-unsaturated aldehydes nearly always give exclusive 1,2-addition (171). With 3-penten-2-one, the ratio of 1,4- to 1,2-addition is 3:1. However, the amount of 1,4-addition of ethylmagnesium bromide to crotonaldehyde drops to zero. Substitution of a second β-methyl group, as in 4-methyl-3-penten-2-one, has the same effect (172). In the same way, the Grignard reaction of Δ^5-7-keto steroid would give only 7-methylcarbinol. Due to the failure of the subsequent Oppenauer oxidation of 7-methylcarbinol (III), this synthetic route was discarded.

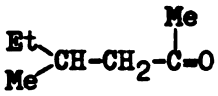


3-Penten-2-one

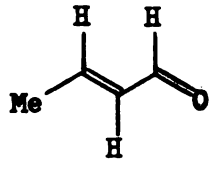
1. EtMgBr
2. H^+, H_2O



25%

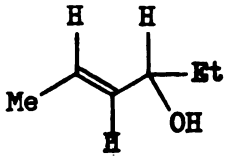


75%

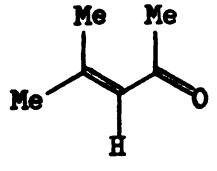


Crotonaldehyde

EtMgBr

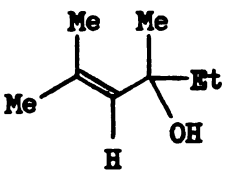


4-Hexen-3-ol
(only product)

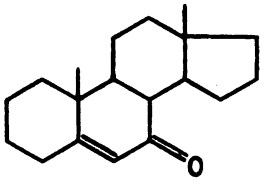


4-Methyl-3-penten-2-one

EtMgBr

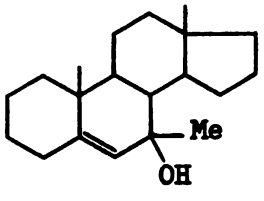


3,5-Dimethyl-4-hexen-3-ol
(only product)



Δ^5-7-keto steroid

MeMgBr



7-Methylcarbinol

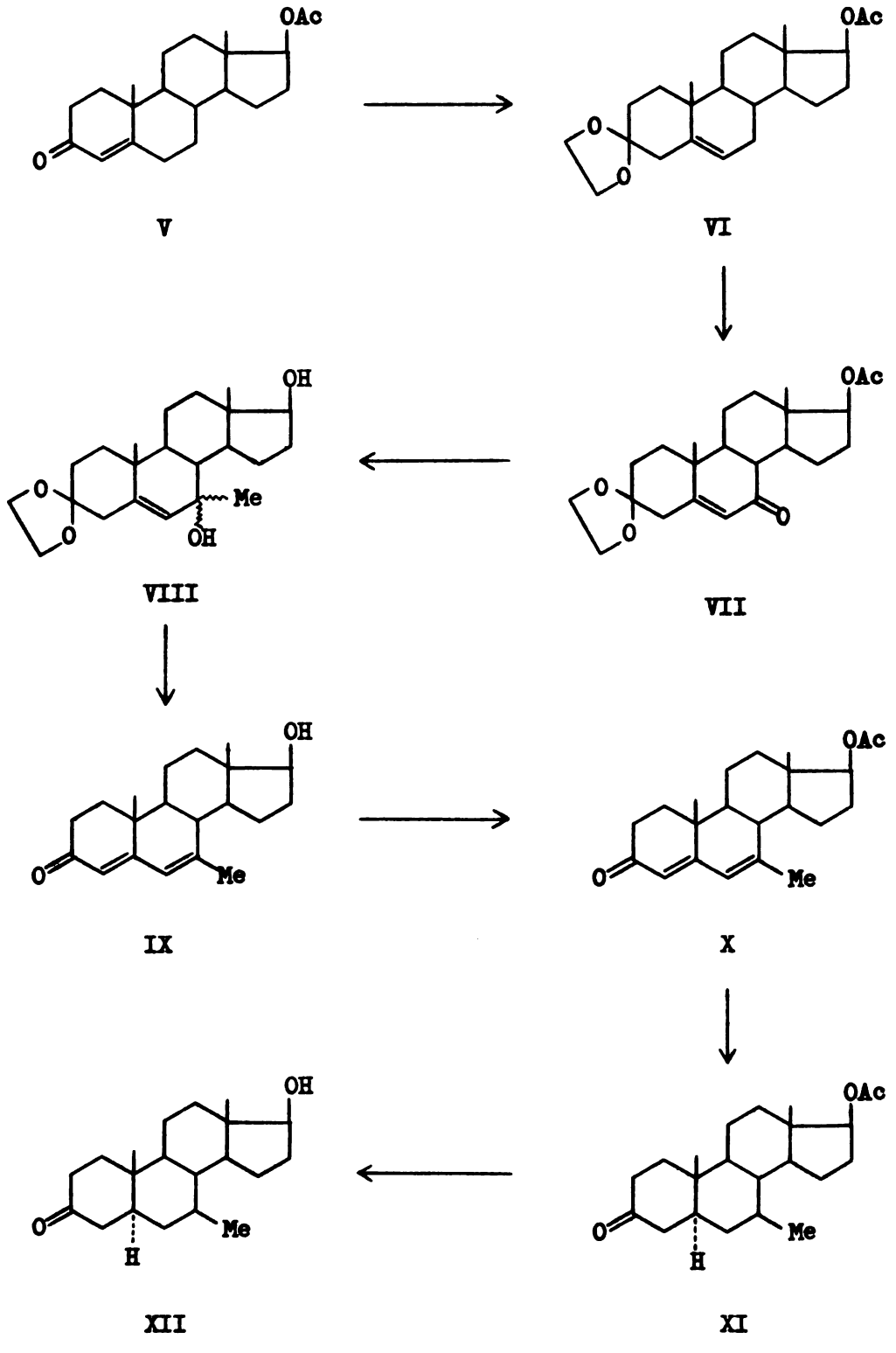
The third synthetic route (shown in Fig. 5) was used. The synthesis of 6-dehydro-7-methyltestosterone (IX) was reported by Zderic et al. (163) using testosterone acetate (V) as starting material. This method was used in the present study.

When testosterone acetate (V) was treated with ethylene glycol in the presence of p-toluenesulfonic acid, the resulting ketal (VI) was formed with concomitant rearrangement of the double bond to the C-5,6 position. Evidence in support of the assigned structure was presented by Fernholz and Stavely (173). After oxidation of VI with t-butyl chromate (167), the 7-keto steroid (VII) was obtained. Rao and Kurath (170) studied the allylic oxidation of VI and isolated the starting material and an α,β-unsaturated ketone with a high ultraviolet absorption maximum at 241 mμ. From this they concluded that the oxidation of VI occurred on the allylic center of C-7 rather of C-4 and the new product was, therefore, formulated as VII. According to Marshall et al. (174), the separation of the 7-keto steroid from the starting material by chromatography on silica gel proved difficult and was only used to prepare analytical samples of the 7-keto steroids. In the present work, however, pure 7-keto product was obtained by recrystallization twice from acetone.

Upon treatment with methylmagnesium bromide, the 7-keto steroid (VII) afforded a mixture of 7-methylcarbinols (VIII). No attempt was made to separate these two epimers since both gave, after hydrolysis and dehydration, 6-dehydro-7-methyltestosterone (IX) in an excellent yield. The corresponding acetate (X) was obtained by treatment of IX with acetic anhydride in pyridine solution.

Hydrogenation of X in the presence of Pd on charcoal in glacial acetic acid containing 10% acetic anhydride gave 7β-methyl-5α-dihydrotestosterone 17-acetate (XI) in an excellent yield. It had a positive CD curve and a positive Cotton effect in the ORD. On this basis it was assigned the 5α-

Fig. 5. Preparation of 7β-methyl-5α-dihydrotestosterone



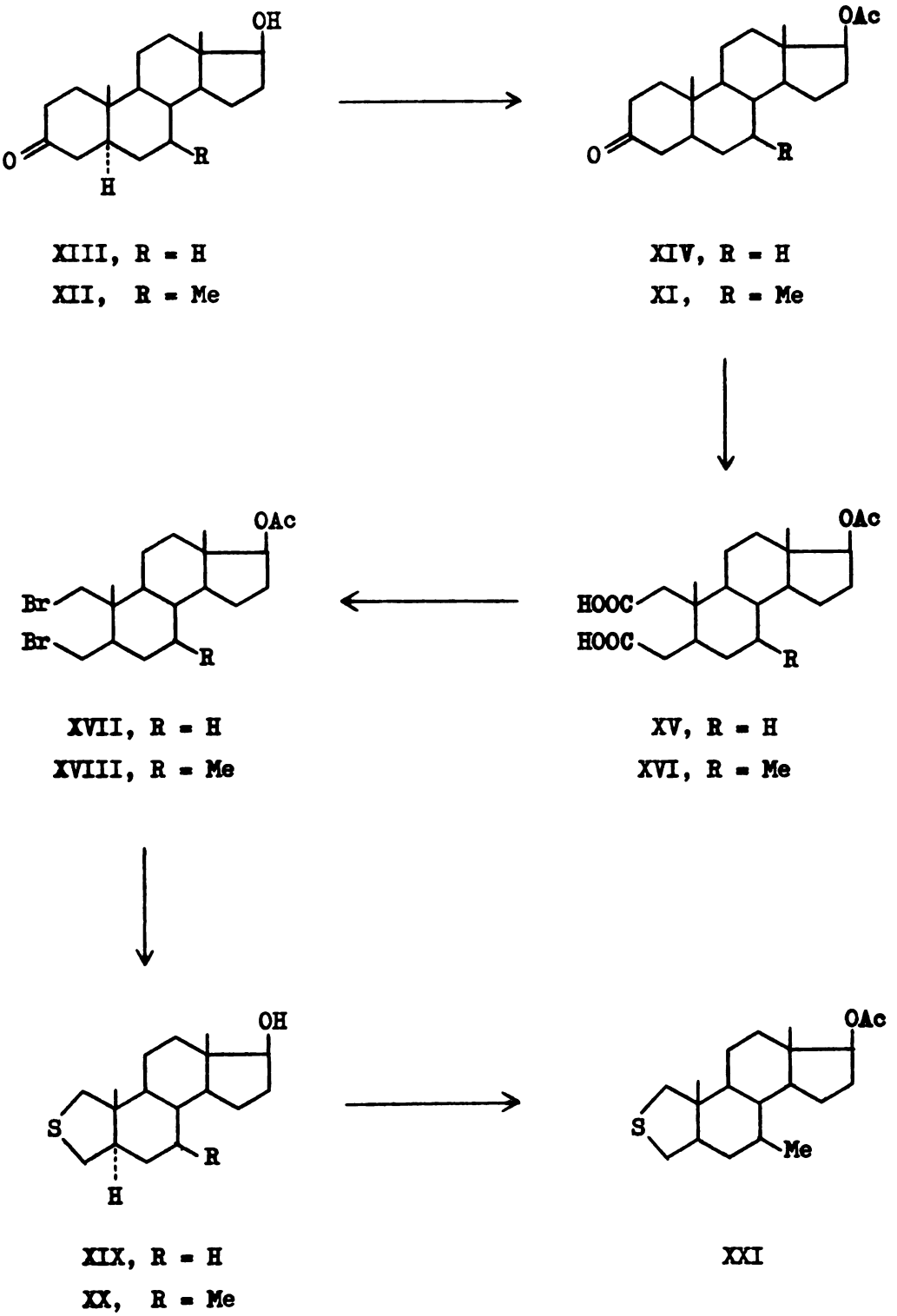
configuration. The assignment of the 7β-configuration was made on the basis of the catalytic hydrogenation of the dienone system in the steroid molecule, which would be expected to proceed by cis-addition of hydrogen to the α-face, and the fact that the compound was not identical to 7α-methyl-5α-dihydrotestosterone. Under similar condition, Beyler et al. (175) obtained 7β-methyl-5α-androstan-3-one derivative in very good yield from the corresponding 7-methyl-4,6-dien-3-one steroid.

The synthesis of 2-thia-A-nor steroids from conventional steroids requires cleavage of the A ring, removal of two carbon atoms, and the obtainment of a dihalide for the incorporation of sulfur atom through a cyclization reaction. This reaction sequence, as shown in Fig. 6, was first accomplished in 1969 by Wolff and Zanati (176). This synthetic route was utilized in the present study to prepare the corresponding 7β-methyl derivatives.

The chromic acid oxidation of ketones generally leads to carbon-carbon bond cleavage with the formation of two carboxylic acids and it has been suggested that the enol is an intermediate in the reaction (177-179). Thus, 5α-androstan-3-one gives 2,3-seco-2,3-dioic acid. The strong preference for a Δ^2-enolization in the 5α-androstan-3-one steroids was difficult to explain. Nevertheless, combustion experiments on 5α-cholest-2-ene and 5α-cholest-3-ene (180) show the former to be more stable by 2.12 Kcal/mole, sufficient to explain a 30:1 preference for the Δ^2-isomer. On the basis of vector analysis calculations, Corey (181) suggested that a Δ^3-double bond forces the 6β-H and 19-methyl group into closer steric opposition, while a Δ^2-double bond can form without increasing this strain. By detailed studies on enolization of 5α-3-ketones including 19-nor, 4α-methyl, and 2α-methyl derivatives, Djerassi (182) has also confirmed the view that both 6β/19 interactions and "hyperconjugation" are operative.

Bromodecarboxylation of the dioic acids, XV and XVI, by the Cristol-

Fig. 6. Preparation of 2-thia-A-nor-5α-androstan-17β-ol and its 7β-methyl derivative



XIII, R = H
XII, R = Me

XIV, R = H
XI, R = Me

XVII, R = H
XVIII, R = Me

XV, R = H
XVI, R = Me

XIX, R = H
XX, R = Me

XXI

Firth modification of the Hunsdiecker reaction (183,184) using the free acid and red mercuric oxide afforded dibmomides, XVII and XVIII, in fairly good yield. Decarboxylative bromination has been reviewed in detail (185-187).

By cyclization in the presence of Na_2S with concomitant cleavage of the protecting group, the dibromides gave the desired 2-thia-A-nor steroids, XIX and XX, in good yield. Acetylation of XX with acetic anhydride in pyridine solution gave 7β-methyl-2-thia-A-nor-5α-androstan-17β-ol acetate (XXI).

(B) Experimental

Melting points were determined with a Thomas-Hoover apparatus equipped with a corrected thermometer. Microanalyses were performed by the Microanalytical Department, University of California, Berkeley. ORD-CD measurements and optical rotations were made with a Jasco ORD/UV-5 apparatus. NMR spectra were obtained at 60 MHz on samples in $CDCl_3$ solution on a Varian A-60 instrument using TMS as the internal standard. The Rf values of 7β-methyl derivatives on TLC were matched with the corresponding 7α-methyl epimers as well as the parent compounds. Androst-5-en-3β,17β-diol diacetate and testosterone acetate were purchased from Searle Chemicals, Inc.

Di-t-butyl chromate -- To 145 ml of t-butanol there was added with stirring 58 g of CrO_3 in small portions. During this period the temperature of the reaction mixture was maitained at 24-25°. After completion of addition, the reaction mixture was stirred for an additional 10 min. The mixture was poured into a separatory funnel and diluted with 400 ml of CCl_4. About 9.5 ml of dark aqueous solution was separated off and the organic solution was taken and dried over anhydrous sodium sulfate. The drying agent was removed by filtration and washed thoroughly with 250 ml of CCl_4. The filtrate was concentrated to about 400 ml by distillation at 40-45° under reduced pressure, whereby the excess t-butanol was azeotropically removed. The CCl_4 solution of di-t-butyl chromate was kept in the refrigerator (167).

3β,17β-Dihydroxyandrost-5-en-7-one diacetate (II) -- A solution of 20 g of androst-5-en-3β,17β-diol diacetate (I) in 200 ml of CCl_4 was warmed to 55°. With vigorous stirring and over 45 min at 55-60°, there was added a solution consisting of 100 ml of acetic acid, 25 ml of acetic anhydride and 150 ml of CCl_4 solution of t-butyl chromate (anhydrous, free of t-butanol and equivalent to 35 g of CrO_3). The reaction mixture was stirred at 60-70° for 20 hr

and then cooled to 20°. The excess chromate was reductively hydrolyzed by the portionwise addition of 950 ml of aqueous 10% oxalic acid, while the temperature was maintained at 20-30°. The mixture was then stirred at room temperature for an additional 2 hr. The pale yellowish CCl_4 layer was separated and the aqueous layer was extracted with $CHCl_3$. The combined organic solutions were washed with water, $NaHCO_3$ solution, water, and dried over anhydrous sodium sulfate. Evaporation of the solvent afforded 20.7 g of white powder which was crystallized from benzene-pet. ether (30-60°) to give 9.6 g of II, mp 219-221° (lit.[167] mp 219-221°). TLC on silica gel showed a single spot with Rf of 0.54 (10% MeOH in $CHCl_3$). The starting material (I) had Rf of 0.60. Upon addition of pet. ether, the mother liquor afforded an additional 4.3 g of the product, mp 216-219°. NMR: 0.83 (s,3, 18-H), 1.23 (s,3, 19-H), 2.04 (s,6, 3,17-di-OAc), 5.75 (s,1, 6-H).

7-Methylandrost-5-en-3β,7,17β-triol (III) -- To a suspension of 5.0 g of II in 250 ml of ether there was added 28 ml of 2.35 M methyllithium in ether over a period of 10 min. After stirring for 2 hr, 30 ml of MeOH was added. The solution was washed 3 times with water, concentrated to about 25 ml, and poured into cold water. The resultant precipitate was collected and dried to yield 3.1 g of crude product. This product was not nicely crystalline, and attempts to purify it failed. It was suitable for use in further reactions.

7-Methylandrost-4,6-dien-3,17-dione (IV) -- A solution of 1.0 g of III and 10 ml of cyclohexanone in 60 ml of toluene was slowly distilled to remove any trace amount of water and 1 g of aluminum t-butoxide was added. After refluxing for 3 hr, the solution was washed with dilute sodium hydroxide and water, dried, and evaporated under reduced pressure to give an oily residue. NMR: 1.01 (s,3, 18-H), 1.13 (s,3, 19-H), 2.04 (s,3, 7-Me), 5.66 (s,1, 6-H),

6.05 (m,1, 4-H). All attempts to purify the product were not successful.

3,3-Ethylenedioxy-17β-hydroxyandrost-5-ene 17-acetate (VI) -- A mixture of 50 g of testosterone acetate (V), 1.2 g of p-toluenesulfonic acid monohydrate, and 350 ml of ethylene glycol (freshly distilled from KOH) in 750 ml of benzene was vigorously stirred and refluxed for 20 hr. About 5.5 ml of water which had formed was collected in a continuous water-removal adapter. After cooling, the mixture was stirred vigorously with aqueous saturated sodium bicarbonate solution for 30 min. The benzene layer was separated, washed twice with water, dried, and evaporated under reduced pressure. The residue was crystallized twice from acetone to give 27.2 g of VI, mp 199-201° (lit.[188] mp 203-205°). TLC on silica gel showed a single spot with Rf of 0.53 (20% acetone in benzene); the starting material (V) had Rf of 0.44. NMR: 0.82 (s,3, 18-H), 1.04 (s,3, 19-H), 1.70 (s,2, 4-H), 2.01 (s,3, 17-OAc), 3.94 (s,4, ketal ethylene). NMR for V: 0.84 (s,3, 18-H), 1.20 (s,3, 19-H), 2.01 (s,3, 17-OAc), 5.71 (s,1, 4-H).

3,3-Ethylenedioxy-17β-hydroxyandrost-5-en-7-one 17-acetate (VII) -- A solution of 41.2 g of VI in 400 ml of CCl_4 was warmed to 55°. With vigorous stirring at 55-60° there was added dropwise a solution consisting of 160 ml of AcOH, 40 ml of Ac_2O, and 400 ml of t-butyl chromate in CCl_4 (anhydrous, free of t-butanol, and equivalent to 58 g of CrO_3). The mixture was stirred at 60-70° for 25 hr and cooled in an ice-bath. A solution of 100 g of oxalic acid dihydrate in 780 ml of water was added slowly with continued cooling and stirring. Then 70 g of solid oxalic acid dihydrate was added and the mixture was stirred at room temperature for an additional 2 hr. The CCl_4 layer was separated and the aqueous layer was extracted with $CHCl_3$. To the combined organic solutions there was added aqueous saturated $NaHCO_3$ solution and the mixture was stirred at room temperature until the formation of CO_2

gas ceased. The organic layer was separated, washed 3 times with water, and dried. After removal of the solvents, 42.8 g of solid was obtained. TLC on silica gel (25% EtOAc in benzene) showed two major spots: one had Rf of 0.25 corresponding to the product (VII); another with Rf of 0.34 unknown. One minor spot had Rf of 0.45 corresponding to the starting material (VI). The residue was crystallized from acetone to give 20 g of product in two crops. TLC still showed the presence of impurity. After recrystallization from the same solvent, 15.8 g of VII was obtained, mp 253-256° (lit.[170] mp 260-261°, purified by chromatography and recrystallized twice from acetone). TLC showed a single spot with Rf of 0.25 (25% EtOAc in benzene) or 0.43 (20% acetone in benzene). NMR: 0.82 (s,3, 18-H), 1.23 (s,3, 19-H), 2.04 (s,3, 17-OAc), 3.97 (s,4, ketal ethylene), 5.7 (d,1, 6-H).

7-Methyl-17β-hydroxyandrost-4,6-dien-3-one (IX) -- To 400 ml of anhydrous tetrahydrofuran (freshly distilled from $LiAlH_4$, bp 65.0-65.5°) containing 15.8 g of VII there was added 150 ml of 3 N methyl magnesium bromide in ether. Because VII was slightly insoluble in tetrahydrofuran at room temperature, the reaction was carried out at 35-40°. After 3.5 hr of stirring, the mixture was poured into aqueous ammonium chloride (with ice). The resultant precipitate was collected, washed with water, and dried to give 15 g of crude product. TLC on silica gel (EtOAc:benzene = 1:1) showed two major spots (Rf 0.06 and 0.15) corresponding to the 7-methylcarbinols (VIII), and one minor spot (Rf 0.22) corresponding to IX. The crude product in 400 ml of 80% aqueous acetic acid solution was heated for 1.5 hr on the steam-bath. The mixture was poured into ice-water and the resultant precipitate collected. It was crystallized from acetone to give 7.1 g of IX, mp 190.5-192° (lit.[163] mp 196-197°). TLC on silica gel showed one spot with Rf of 0.22 (EtOAc: benzene = 1:1) or 0.23 (20% acetone in benzene). NMR: 0.86 (s,3, 18-H), 1.09 (s,3, 19-H), 1.91 (s,3, 7-Me), 5.59 (s,1, 6-H), 5.96 (s,1, 4-H). The

residue from the mother liquor gave, after crystallization from EtOAc, an additional 1.95 g of IX, mp 190-191.5°.

7-Methyl-17β-hydroxyandrost-4,6-dien-3-one 17-acetate (X) -- A solution of 7.3 g of IX in 50 ml of pyridine was treated with 8 ml of acetic anhydride and kept at room temperature for 22 hr. The mixture was poured into water and the resultant precipitate (7.9 g) was collected. It was crystallized from hexane to give 6.5 g of X, mp 136-138° (lit.[163] mp 139-141°). By concentration of the mother liquor, an additional 0.64 g of X was obtained, mp 132-134°. TLC on silica gel showed a single spot with Rf of 0.41 (20% acetone in benzene). NMR: 0.93 (s,3, 18-H), 1.10 (s,3, 19-H), 1.93 (s,3, 7-Me), 2.05 (s,3, 17-OAc), 5.62 (s,1, 6-H), 5.99 (m,1, 4-H).

7β-Methyl-17β-hydroxy-5α-androstan-3-one 17-acetate (XI) -- To a solution of 4.1 g of X in 136 ml of acetic acid and 14 ml of acetic anhydride there was added 150 mg of 10% Pd-C. The mixture was hydrogenated at 600 mmHg initial hydrogen pressure (Parr low pressure hydrogenator) for 1 hr at room temperature. TLC indicated the completion of hydrogenation. The catalyst was removed by filtration and the solvents were evaporated under reduced pressure. The oily residue was chromatographed on silica gel, using 4-5% acetone in benzene as eluent, to give 3.4 g of XI, mp 126-128°. TLC showed a single spot with Rf of 0.53 (20% acetone in benzene). NMR: 0.83 (s,3, 18-H), 1.01 (s,3, 19-H), 2.03 (s,3, 17-OAc). It was crystallized from ethanol to give the analytical sample, mp 128-130°; ORD (c 0.4, EtOH), 20°; $[\phi]_{350}$ +2080°, $[\phi]_{308}$ +4590°, $[\phi]_{284}$ 0°, $[\phi]_{270}$ -1040°, $[\phi]_{249}$ 0°; CD, $[\theta]_{292}$ +3860.

Anal. Calcd. for $C_{22}H_{34}O_3$: C, 76.26; H, 9.89. Found: C, 76.30; H, 9.89.

7β-Methyl-17β-hydroxy-5α-androstan-3-one (XII) -- A solution of 0.050 g of XI in 5 ml of 5% KOH-Methanol solution was stirred at room temperature

for 2 hr when TLC showed the completion of hydrolysis. The reaction mixture was poured into water and the resultant precipitate was collected, dried and crystallized from EtOAc-hexane to give 0.04 g of XII, mp 153-155°. NMR: 0.78 (s,3, 18-H), 1.01 (s,3, 19-H). Further crystallization from the same solvent afforded the analytical sample, mp 157-159°.

Anal. Calcd. for $C_{20}H_{32}O_2$: C, 78.89; H, 10.60. Found: C, 78.62; H, 10.41.

17β-Hydroxy-5α-androstan-3-one 17-acetate (XIV) -- A solution of 10 g of 5 -dihydrotestosterone (XIII) in 60 ml of pyridine and 10 ml of acetic anhydride was allowed to react at room temperature for 20 hr. The mixture was poured into ice-water and the resultant precipitate was collected, washed with water, and dried. It was crystallized from methanol to give 11 g of XIV, mp 155-157° (lit.[189] mp 157-158°).

17β-Hydroxy-2,3-seco-5α-androstane-2,3-dioic acid 17-acetate (XV) -- To a stirred solution of 6 g of XIV in 120 ml of glacial acetic acid at 54° there was added dropwise a solution of 6 g of CrO_3 in 18 ml of water and 18 ml of acetic acid. The mixture was stirred at 60-61° for 20 hr and poured into ice-water. The resultant precipitate was collected, washed with water, and dried to give 4.5 g of pale green powder, mp 208-214°. Crystallization from EtOAc gave 2.7 g of XV, mp 225-227° (lit.[190] mp 223-225°).

7β-Methyl-17β-hydroxy-2,3-seco-5α-androstane-2,3-dioic acid 17-acetate (XVI) -- To a stirred solution of 2.08 g of XI in 40 ml of glacial acetic acid at 55° there was added a solution of 2.0 g of CrO_3 in 6.0 ml of water and 6.0 ml of acetic acid. The mixture was stirred at 60° for 7 hr. TLC showed the completion of the reaction. The mixture was poured into ice-water saturated with NaCl and the resultant precipitate was collected and washed with water. The pale greenish white solid was taken up in aqueous $NaHCO_3$ solution and the aqueous solution was washed with ether. The combined

ether washings were extracted once with aqueous $NaHCO_3$ solution. The combined aqueous solution was cooled, saturated with NaCl, and acidified with concd. HCl. The white precipitate was collected, washed with water, and dried to give 2.07 g of powder. It was crystallized from aqueous methanol to give 1.15 g of XVI, mp 208-211°. TLC showed one spot with Rf of 0.24 (10% MeOH in $CHCl_3$). Recrystallization from EtOAc gave the analytical sample, mp 212-214°, $[\alpha]^{20}D$ +28° (c 1, 95% EtOH).

Anal. Calcd. for $C_{22}H_{34}O_6$: C, 66.98; H, 8.69. Found: C, 66.76; H, 8.43.

1,4-Dibromo-1,4-seco-2,3-bisnor-5α-androstan-17β-ol acetate (XVII) -- A suspension of 2.7 g of XV and 2.2 g of red HgO in 150 ml of CCl_4 was refluxed with stirring. The mixture was shielded from the light and 2.2 g of Br_2 (ca 0.8 ml) was added dropwise. After 3 hr, the solvent was removed under reduced pressure, and the residue extracted with CCl_4. The combined extracts were filtered and evaporated. The pale yellowish residue was crystallized from methanol to give 2.2 g of product, mp 145-155°. Recrystallization from the same solvent afforded 1.66 g of XVII, mp 157-160° (lit.[176] mp 155-158°). TLC on silica gel showed one spot with Rf of 0.64 (25% EtOAc in benzene).

7β-Methyl-1,4-dibromo-1,4-seco-2,3-bisnor-5α-androstan-17β-ol acetate (XVIII) -- A suspension of 1.74 g of XVI (ground to a very fine powder) and 1.45 g of red HgO in 100 ml of CCl_4 was shielded from the light and refluxed with stirring. To the mixture there was slowly added 1.42 g of Br_2 (ca 0.50 ml) and the reaction mixture was refluxed for an additional 3 hr. After cooling, the mixture was filtered and the filtrate evaporated under reduced pressure to give an oily residue. After purification with column chromatography on silica gel, using 3-4% acetone in pet. ether (30-60°) as eluent, 1.5 g of the product (XVIII) was obtained, mp 120-123°. TLC on

silica gel showed one spot with Rf of 0.64 (25% EtOAc in benzene), or 0.45 (20% acetone in hexane), or 0.60 (10% MeOH in $CHCl_3$). It was crystallized from 95% EtOH to give the analytical sample, mp 123-125°, $[\alpha]^{20}D$ +0.7° (c 1, $CHCl_3$).

Anal. Calcd. for $C_{20}H_{32}O_2Br_2$: C, 51.73; H, 6.95; Br, 34.42. Found: C, 51.60; H, 6.87; Br, 34.2.

2-Thia-A-nor-5α-androstan-17β-ol (XIX) -- To a refluxing solution of 1.5 g of XVII in 100 ml of ethanol there was added a tenfold excess of $Na_2S \cdot 9H_2O$ in the minimum amount of hot water. The mixture was refluxed for 18 hr when TLC indicated the complete conversion of the dibromide to the product. The solvent was evaporated under reduced pressure and the solid residue was taken up in ether, washed several times with water, dried, and evaporated to give 0.98 g of white powder, mp 139-141°. Crystallization from hexane afforded XIX, mp 140-142° (lit.[176] mp 141-143°).

7β-Methyl-2-thia-A-nor-5α-androstan-17β-ol (XX) -- This compound was synthesized from 0.138 g of XVIII in a manner similar to that described above. The crude product weighed 0.077 g, mp 150-155°. TLC on silica gel showed one spot with Rf of 0.40 (25% EtOAc in benzene), or 0.23 (20% acetone in hexane). It was crystallized from hexane to give the analytical sample, mp 162-164°, $[\alpha]^{20}D$ +84° (c 1, $CHCl_3$).

Anal. Calcd. for $C_{18}H_{30}OS$: C, 73.41; H, 10.27; S, 10.89. Found: C, 71.65; H, 9.56; S, 10.56.

7β-Methyl-2-thia-A-nor-5α-androstan-17β-ol acetate (XXI) -- A solution of 0.70 g of crude XX in 10 ml of pyridine and 0.7 ml of acetic anhydride was allowed to stand at room temperature for 44 hr. TLC showed one spot with Rf of 0.58 (25% EtOAc in benzene), or 0.48 (20% acetone in hexane), corresponding to the product (XXI). The mixture was poured into water and

extracted with ether. The combined extracts were washed with dil. HCl, water, dried, and evaporated. The oily residue was chromatographed on silica gel, using 3-4% acetone in pet. ether (30-60°) as eluent, to give a white crystalline product. It was crystallized from pet. ether (30-60°) to afford 0.42 g of XXI, mp 75-77°. Further crystallization from ethanol gave the analytical sample, mp 77-79°, $[\alpha]^{20}D$ +75° (c 1, $CHCl_3$).

Anal. Calcd. for $C_{20}H_{32}O_2S$: C, 71.38; H, 9.59; S, 9.53. Found: C, 71.29; H, 9.59; S, 9.40.

PART IV

BIOLOGICAL EVALUATION

(A) Pharmacological testing

Androgenic-anabolic tests (22,191) -- The test compounds in carboxymethocellulose (CMC) solution were given by subcutaneous administration once daily for seven days to castrate male rats 21 days of age at the start of the test. Autopsy was performed on the day following the last day of administration. A total dose of 3 mg/rat of testosterone propionate was used as a standard. The results of the test are shown in Table 2.

Testing for anti-tumor activity (192) -- The 13762 mammary adenocarcinoma, originally DMBA (7,12-dimethylbenzanthracene) induced, is 100% transplantable and lethal in syngeneic Fischer 344 strain female rats (see Refs. 193-197 for induction of breast cancers by polynuclear aromatic hydrocarbons or aromatic amines). On day 0, routine size (1-2 mm^3) grafts of the 13762 mammary tumor were implanted, subcutaneously, right side, into 19 Fischer 344 strain females at approximately 40 days of age. Due to limited quantities of test materials only one dose level (10 mg/Kg/day) and a small number of test animals were used. Treatment was initiated on day 1 and continued daily for 20 consecutive days. Test compounds were formulated in sesame oil and administered intraperitoneally. The control group was administered only the sesame oil vehicle at 0.5 ml/day. Twenty-four hours after the last treatment all animals were sacrificed for the various endpoint determinations: IBW (initial body weight), FBW (final body weight minus tumor weight), TW (tumor weight), FTS (final tumor size), spleen weight, ovarian weight, uterine weight, adrenal weight, thymus weight. The results of the test are shown in Table 3.

Table 2. Androgenic-Anabolic Tests

Treatment Total Dose		Body Weight Initial (gm)	 Final (gm)	Ventral Prostate (mg)	Seminal Vesicles (mg)	Levator Ani (mg)
Controls		52	85	18.5	13.5	26.5
CMC		52	85	17.9	13.8	24.0
		54	89	17.9	13.0	23.4
		55	89	17.3	14.1	19.8
		56	91	16.1	13.8	24.2
	Ave.	54	88	17.5	13.6	23.6
	S.E.±	0.8	1.2	0.41	0.19	1.08
Testosterone		54	90	141.3	71.5	69.5
propionate		56	102	124.5	62.5	66.3
3 mg		56	95	135.0	68.2	60.3
		58	105	116.3	53.5	70.5
		56	100	113.0	74.2	74.3
	Ave.	56	98	126.0	65.9	68.2
	S.E.±	0.6	2.7	5.4	3.7	2.4
7β-Methyl-5α-DHT 17-OAc		53	89	74.3	39.2	58.8
3 mg		55	85	72.7	47.1	60.5
		56	93	89.0	57.1	69.0
		56	95	81.1	40.0	62.5
		56	90	89.8	45.6	65.7
	Ave.	55	90	81.4	45.8	63.3
	S.E.±	0.6	1.7	3.6	3.2	1.8
7β-Methyl-2-thia-		59	93	24.5	12.2	33.5
A-nor-5α-androstan-		59	89	17.2	11.5	22.5
17β-ol acetate		55	90	19.0	13.0	22.9
3 mg		59	91	16.3	11.8	25.6
		56	88	16.0	10.5	24.0
	Ave.	58	90	18.6	11.8	25.7
	S.E.±	0.9	0.9	1.6	0.4	2.0
5α-DHT		56	95	85.8	50.5	46.5
1 mg		55	89	102.3	57.3	68.2
		56	82	108.0	51.0	60.5
		53	93	70.5	36.0	46.5
		58	95	106.2	57.5	61.8
	Ave.	56	91	94.6	50.5	56.7
	S.E.±	0.8	2.5	7.2	3.9	4.4

Table 2. (continued)

Treatment Total Dose		Body Weight Initial (gm)	Body Weight Final (gm)	Ventral Prostate (mg)	Seminal Vesicles (mg)	Levator Ani (mg)
5α-Androst-2-en-17β-ol 1 mg		54	100	47.5	28.1	49.0
		58	94	42.5	23.4	61.1
		55	96	49.2	25.8	57.8
		58	96	42.4	24.2	66.5
		56	104	50.5	29.0	63.8
	Ave.	56	98	46.4	26.1	59.6
	S.E.±	0.8	1.8	1.7	1.1	3.0
5α-Androst-2-en-17β-ol 3 mg		54	97	90.5	51.5	66.3
		54	93	85.5	58.0	71.0
		56	98	98.2	62.8	72.1
		55	95	107.5	66.3	67.9
		52	93	100.4	53.5	76.5
	Ave.	55	95	96.4	58.4	70.8
	S.E.±	0.4	1.0	3.8	2.8	1.8
5α-Androst-2-en-17β-ol 6 mg		55	95	119.6	71.5	71.3
		60	98	109.1	69.5	70.4
		57	94	88.5	75.5	70.6
		58	96	99.0	70.5	75.8
		59	89	114.0	78.3	68.3
	Ave.	58	94	106.0	73.1	71.3
	S.E.±	0.9	1.5	5.5	1.7	1.2

Table 3. Testing for anti-tumor activity

	Group I Sesame Oil	Group II 7β-Methyl-DHT 17-acetate	Group III 7β-Methyl-2-thia-A-nor steroid
Dose Level (mg/Kg/day)	0.5 ml	10.0	10.0
Route	I.P.	I.P.	I.P.
No. Animals $\frac{\text{Surviving}}{\text{Treated}}$	10/10	4/4	5/5
IBW/FBW[a]	1.56	1.58	1.50
$\frac{\text{Organ Wt.}}{\text{100 g FBW minus Tumor Wt.}}$			
Tumor Wt. (mg)	6.65 ± 1.91	5.58 ± 2.39	5.64 ± 1.81
% Control		84%	85%
		p > 0.1	p > 0.1
Spleen Wt. (gm)	0.43 ± 0.14	0.33 ± 0.05	0.35 ± 0.06
		77%	81%
		p > 0.1	p > 0.1
F.T.S.[b] (L+W/2mm)	29.6 ± 3.3	26.6 ± 3.6	25.3 ± 3.4
		90%	85%
		p > 0.1	p < 0.05
Ovarian Wt. (mg)	55.5 ± 7.1	42.6 ± 6.8	58.2 ± 11.2
		77%	105%
		p < 0.01	p > 0.1
Uterine Wt. (mg)	122.5 ± 16.4	174.9 ± 46.8	152.5 ± 51.4
		143%	124%
		p < 0.01	p > 0.1
Adrenal Wt. (mg)	39.9 ± 6.5	27.3 ± 5.9	40.3 ± 4.8
		68%	101%
		p < 0.01	p > 0.1
Thymus Wt. (mg)	176.0 ± 61.8	124.6 ± 42.9	220.7 ± 42.3
		71%	125%
		p > 0.1	p > 0.1

a. Initial Body Weight/Final Body Weight Ratio

b. Final Tumor Size (Length + Width/2 mm)

(B) Results and discussion

Many 7α-methyl steroids (Table 4) and few 7β-methyl steroids (Table 1) have been studied and tested for their pharmacological activities. In general, 7α-methyl substitution increases both androgenic and anabolic potencies, giving rise very often to a favorable anabolic-androgenic ratio whereas 7β-methyl substitution decreases both activities to a very large degree. In the present work, the pharmacological testing (Table 2) showed that 7β-methyl-5α-dihydritestosterone acetate was only weakly androgenic whereas 7β-methyl-2-thia-A-nor-5α-androstan-17β-ol was devoid of androgenic activity. These results are well in harmony with those earlier findings.

The difference in potency between the DHT and the 2-thia-A-nor steroid series might be a combined effect on receptor binding affinity, intrinsic activity, and other factors, such as the rates of uptake and transformation from the sites of administration to the target tissues or the distribution and the metabolic stability in whole animals. However, as previously pointed out by Wolff et al. (72,73), if a group like 7α-methyl enhances androgenic and anabolic activities in both the testosterone series (as well as DHT and 19-nortestosterone series) and the 2-thia-A-nor steroid series, it is reasonable to conclude that this effect is mediated at the receptor affinity-intrinsic activity level, and not through changes in drug distribution or drug metabolism. Similarly, since the introduction of 7β-methyl group decreases both androgenic and anabolic activities in both the testosterone series (as well as DHT series) and the 2-thia-A-nor steroid series, this effect also must be mediated at the receptor affinity-intrinsic activity level.

In view of the large increases in the androgenic and anabolic potencies of the 7α-methyl substituted compounds, α-face attachment of the receptor at C-7 is not involved (71). On the contrary, the increase in activity is most

Table 4. 7α-Methyl substituted androgens

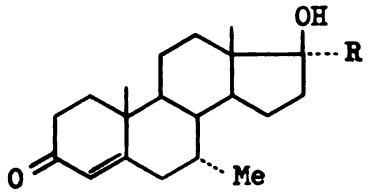
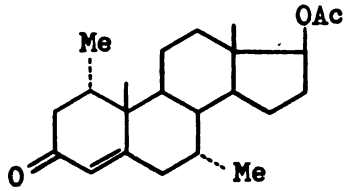
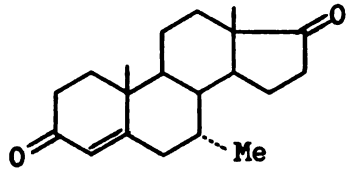
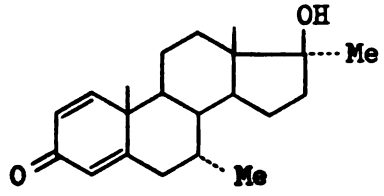
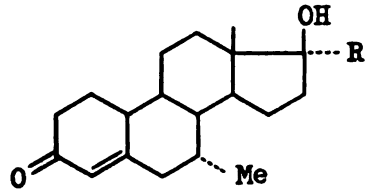
Structure	Name	Ref.
	Testosterone series:	
	R = H: 7α-Methyltestosterone	19,70
	R = Me: 7α,17α-Dimethyltestosterone	19,70 198,199
	1α,7α-Dimethyltestosterone 17-acetate	164
	7α-Methylandrost-4-en-3,17-dione	19
	7α,17α-Dimethyl-17β-hydroxy-androst-1,4-dien-3-one	200
	19-Nortestosterone series:	
	R = H: 7α-Methyl-19-nortestosterone and its 17-acetate	70,198 201
	R = Me: 7α,17α-Dimethyl-19-nor-testosterone	19,70 198,201
	R = C≡CH: 7α-Methyl-17α-ethynyl-19-nortestosterone	202

Table 4. (continued)

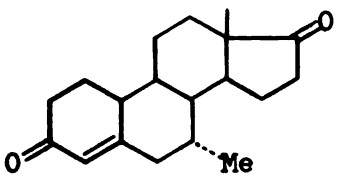
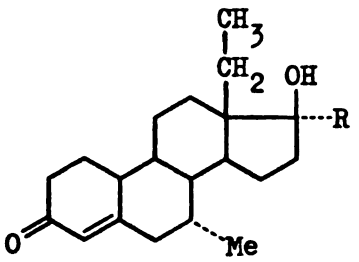
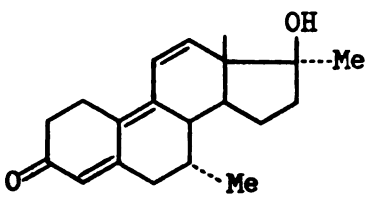
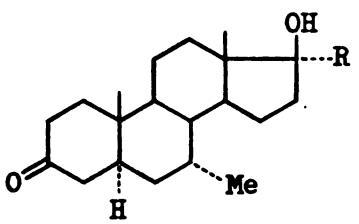
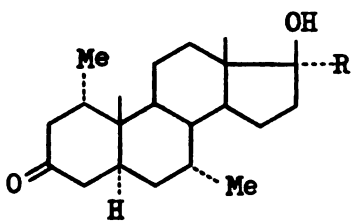
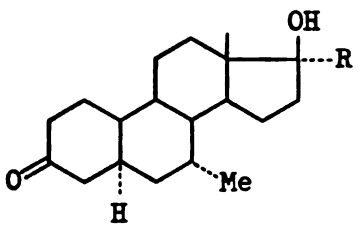
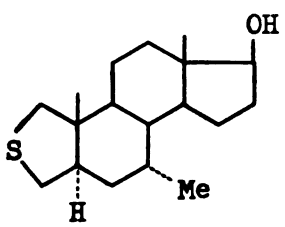
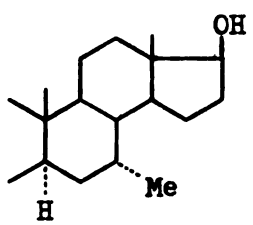
Structure	Name	Ref.
	7α-Methylestr-4-en-3,17-dione	19
	R = H: 13β-Ethyl-17β-hydroxy-7α-methylgon-4-en-3-one	203
	R = Et: 13β,17α-Diethyl-17β-hydroxy-7α-methylgon-4-en-3-one	203
	R = C≡CH: 13β-Ethyl-17α-ethynyl-17β-hydroxy-7α-methylgon-4-en-3-one	203
	7α,17α-Dimethyl-17β-hydroxyestra-4,9,11-trien-3-one	204
	5α-Dihydrotestosterone series:	
	R = H: 7α-Methyl-5α-dihydrotestosterone	70, 205
	R = Me: 7α,17α-Dimethyl-5α-dihydrotestosterone	70
	R = H: 1α,7α-Dimethyl-5α-dihydrotestosterone and its 17-acetate	164
	R = Me: 1α,7α,17α-Trimethyl-5α-dihydrotestosterone	164

Table 4. (continued)

Structure	Name	Ref.
	19-Nor-5α-dihydrotestosterone series:	
	R = H: 7α-Methyl-19-nor-5α-dihydrotestosterone	70
	R = Me: 7α,17α-Dimethyl-19-nor-5α-dihydrotestosterone	70
	2-Thia-A-nor steroid series:	
	7α-Methyl-2-thia-A-nor-5α-androstan-17β-ol and its acetate	72
	Nonsteroid series:	
	7α-Methyl-1,4-seco-2,3-bisnor-5α-androstan-17β-ol	11

probably due to a change produced in the conformation of the steroid itself, through conformational transmission (72-74). As described before, the axial 7α-methyl is involved in repulsive interactions with the 5α,9α, and 14α protons. Therefore, the effect of this substituent would be to flatten the molecule toward the β-face which favors the hypothesis of the steroid-receptor complex proposed by Wolff et al. (60). On the other hand, since the equatorial 7β-methyl is in the plane of the steroid molecule, an influence on α-face or β-face attachment of the receptor at C-7 is probably not involved. The large decrease in the androgenic and anabolic potencies of 7β-methyl substituted compounds is thus probably mediated through direct interaction of the substituent with the receptor surface in contact with the third-dimension (or front side) of the steroid molecule.

The 7β-methyl group is very important in connection with the anti-tumor activity since introduction of this group into testosterone (e.g. Calusterone) largely enhanced the anti-tumor efficacy in the treatment of advanced female breast cancer while decreasing the androgenic activity (53). The results of testing for anti-tumor activity of compounds 7β-methyl-5α-dihydrotestosterone acetate and its 2-thia-A-nor analog are summerized in Table 3. The initial body weight/final body weight ratio indicates no real toxicity at the dose levels used. Reduction in tumor weight was not significant for either compound but the final tumor size in the animals treated with 7β-methyl-2-thia-A-nor steroid was significantly reduced. From the lack of toxicity, it may well be that, at the dose levels used, the results obtained were at the bottom of a dose response curve and that 3 to 4 times that concentration of compound could have been tolerated.

Of particular interest are the effects on the various organ weights. Tumor growth is a stressful effect and one generally sees some adrenal hypertrophy, thymolysis, and loss of spleen weight in mammary tumor-bearing animals.

At the dose levels used both test compounds had an estrogenic effect on the uterus with 7β-methyl-DHT exhibiting greatest activity. Animals treated with 7β-methyl-DHT had significantly smaller ovaries. The 7β-methyl-2-thia-A-nor steroid appeared to have a protective effect on the thymus in contrast to 7β-methyl-DHT.

In the present study, due to limited quantities of test compounds available only one dose level (10 mg/Kg/day) and a small number of test animals were used. Since toxic effects were not evident and only one dose level was used, there is little question that larger doses could be safely tolerated. Based upon the limited results of this study the determination of the tumor-inhibitory action of 7β-methyl-DHT and its 2-thia-A-nor analog would have to be carried out at dose levels of about 50 mg/Kg/day and a larger number of test animals.

PART V

EFFECT OF STEROIDS ON RETENTION OF ^{3}H-5α-DHT BY RAT VENTRAL PROSTATE IN VITRO AND IN VIVO

(A) Materials and experimental procedures.

Materials -- 1,2-^{3}H-5α-Dihydrotestosterone (17β-Hydroxy-5α-androstan-3-one, 44 Ci/mMole) was purchased from New England Nuclear. Following evaporation of the organic solvents (ethanol and benzene), ^{3}H-DHT were dissolved in saline containing 10% EtOH, and diluted to a concentration of 100 uCi/mMole. The purity was checked by thin layer chromatography and no radiochemical impurities were found. 5α-Dihydrotestosterone was purchased from Searle Chemicals. It was crystallized once from ethyl acetate, mp 179-181°. Anavar (17β-Hydroxy-17α-methyl-2-oxa-5α-androstan-3-one; oxandrolone) and Nilevar (17α-Ethyl-19-nortestosterone) were obtained as a gift from Searle Chemicals. Halotestin (9α-Fluoro-11β-hydroxy-17α-methyltestosterone; Fluoxymesterone) was obtained as a gift from the Upjohn Company. Winstrol (17β-Hydroxy-17α-methyl-5α-androstano[3,2-c]pyrazole; Stanozolol) was obtained as a gift from Sterling-Winthrop Research Institute. Cyproterone (6-Chloro-17α-hydroxy-1α,2α-methylene-pregna-4,6-dien-3,20-dione) was a gift from Dr. U. Kerb, Schering A-G, Berlin. 7α-Methyl-5α-dihydrotestosterone and 7α-methyl-2-thia-A-nor-5α-androstan-17β-ol were prepared by Dr. Gunhild Gaare. Eagle's minimum essential medium (with Earle's salts) and L-glutamine were purchased from Grand Island Biological Company. PPO (2,5-Diphenyloxazole), scintillation grade, and POPOP (p-bis[2-(5-phenyloxazolyl)]-benzene), scintillation grade, were purchased from New England Nuclear.

Treatment of Animals -- Adult male Sprague-Dawley rats weighing 300-350 g were used throughout this study. They were bilaterally orchidectomized one day before the experiment started. They were given standard laboratory diet and water.

Incubation of Minced Prostate -- Ventral prostate excised from castrate rats was minced with scissors and approximately 30-40 mg of the prostate minces was incubated in a 25-ml flask containing 5.0 ml of Eagle's minimum essential medium (containing 2.5% EtOH), ^{3}H-5α-DHT (9.2×10^{-10}M) and non-radioactive steroid (3.4×10^{-6}M) under an atmosphere of air at 37° for either 30 min or 2 hr. Right before the experiment started, 10 mM of L-glutamine was added to 100 ml of Eagle's minimum essential medium. Care was taken in order to get practically the same amount of tissue in each flask. The incubations were carried out during continuous shaking (65 oscillations per min). Following incubation, the prostate tissue was gently blotted with filter paper and washed in a steroid-free Eagle's minimum essential medium at 25° for 30 min with a shaking rate of 65 oscillations per min. After the incubation-washing procedure, the tissue was cooled in ice-water and gently dried on a filter paper in order to remove any surface moisture, and transferred to preweighed scintillation vials. The wet tissue weights were obtained by difference. To each vial about 2 ml of $CHCl_3$-CH_3OH (2:1) was added and the vials were left in the refrigerator overnight to extract the radioactive component. The solvents were evaporated to dryness and the samples were subjected to scintillation spectrometry to determine radioactivity.

Measurement of Radioactivity -- To each polyethylene scintillation vial containing radioactive sample was added 10 ml of scintillation liquid consisting of 5 g of PPO and 300 mg of POPOP in 1,000 ml of scintillation grade toluene. The radioactivity was determined by Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3375. Quenching correction was obtained by the external standard ratio method. The counting efficiency was about 35%.

Solubility of Steroids in Aqueous Solution -- In general, steroids are very insoluble in aqueous solution and, in the competition binding experi-

ments, the addition of large excess amount of nonradioactive steroid to the incubation system may affect the solubility of radioactive steroid in the solution. It is, therefore, very important to determine whether or not the radioactivity in each sample maintains the same level before incubation. In order to do this, to the same volume of water (containing 2.5% EtOH) as used in the competition experiments the same amount of radioactive and nonradioactive steroids were added. The mixture was agitated with a Vortex stirrer for 30 sec and filtered. The radioactivity in the filtrates was then determined by the procedure described above.

In vivo competition -- Each castrate rat was given 2 mg of nonradioactive steroid dissolved in 0.3 ml of EtOH by deep intramuscular injection into the thigh. Fifteen min later 30 μCi of ^{3}H-DHT in a saline solution containing 15% EtOH was given. The rats were sacrificed 30 min after the last injection. The ventral prostate were then dissected free of fat and adhering connective tissues, washed in saline solution for 30 min. Care was taken to exclude possible contamination with radioactive urine and blood. The tissue was gently dried on a filter paper in order to remove any surface moisture, and transferred to preweighed scintillation vials. The tissue weights were obtained by difference. The tissue was chopped with scissors, kept in a $CHCl_3$-CH_3OH (2:1) solution and left in the refrigerator overnight to extract the radioactive component. The solvents were evaporated to dryness and the samples were subjected to scintillation spectrometry to determine radioactivity.

Table 5. Retention of ^{3}H-5α-DHT by minced ventral prostate.

Exp.	Castration (day)	Incubation time (min)	^{3}H-DHT (cpm/mg)	^{3}H-DHT with 5α-DHT (cpm/mg)	Inhibition of radioactivity (%)
1	Intact	120	255.3	239.4	6.2
2	1	120	253.6	179.0	29.4
3	1	120	309.8	210.6	32.0
4	3	120	229.8	143.3	37.6
5	1	30	205.2	131.2	36.1
6	1	30	277.3	173.8	37.3
7	1	30	293.7	186.7	36.4
8	1	30	271.8	183.5	32.5
9	1	30	269.2	185.7	31.0
10	1	30	260.1	166.0	36.2

a. Results are mean values of two determinations.

b. In each experiment 4 to 5 rats were used.

Table 6. Effect of nonradioactive steroids on retention of radioactivity by minced ventral prostate incubated with ^{3}H-DHT at 37° for 30 min.

Nonradioactive steroid added	Inhibition of radioactivity uptake (%) Exp. 1	Exp. 2
DHT	33.7	32.3
7α-Methyl-DHT	19.7	18.7
7β-Methyl-DHT	29.3	26.4
Cyproterone	34.6	36.4
DHT	36.1	36.4
2-Thia-A-nor-5α-androstan-17β-ol	39.6	45.8
7α-Methyl-2-thia-A-nor steroid	42.7	49.8
7β-Methyl-2-thia-A-nor steroid	36.1	34.2
5α-Androst-2-en-17β-ol	-	37.8
DHT	32.5	37.3
Anavar	27.8	27.8
Halotestin	36.2	44.1
Nilevar	40.1	40.2
Winstrol	38.6	41.9
DHT	36.2	
DHT 17-acetate	17.5	
7β-Methyl-2-thia-A-nor-5α-androstan-17β-ol	28.5	
7β-Methyl-2-thia-A-nor-5α-androstan-17β-ol acetate	0	

a. Results are mean values of two determinations.

b. In each experiment 4 to 5 rats one day after castration were used.

Table 7. Effect of nonradioactive steroids on retention of radioactivity by minced ventral prostate incubated with ^{3}H-DHT at 37° for 120 min.

Nonradioactive steroid added	Inhibition of radioactivity uptake (%) Intact rat Exp. 1	One-day castrated rat Exp. 2	Exp. 3
DHT	6.2	32.0	29.4
7α-Methyl-DHT	9.4	47.6	50.9
7β-Methyl-DHT	4.9	17.3	17.3
Cyproterone	-	-	28.5

a. Results are mean values of two determinations.

b. In each experiment 4 to 5 rats were used.

Table 8. Effect of nonradioactive steroids on the solubility of ^{3}H-DHT in water.

^{3}H-DHT (1.25 μg/ml) and nonradioactive steroid	μg/ml	Precipitation observed	cpm/ml	Inhibition of radioactivity (%)
None (control)				
in EtOH		-	285,504	
in water		-	281,039	
DHT	12.5	+	235,425	16.23
DHT	25.0	+	210,285	25.18
5α-Androst-2-en-17β-ol	25.0	+	253,800	9.69
2-Thia-A-nor-5α-androstan-17β-ol	25.0	+	223,753	20.38
5α-Androstan-17β-ol	25.0	+	256,690	8.67
Cyproterone	25.0	-	288,017	0

Table 9. Solubility of steroids in water.

^{3}H-5α-DHT (9.2×10^{-10} M) with nonradioactive steroid (3.4×10^{-10} M)	cpm/ml	Inhibition of radioactivity (%)
Control	38,026	
5α-DHT	37,739	0.8
7α-Methyl-5α-DHT	37,887	0.4
7β-Methyl-5α-DHT	37,760	0.7
Cyproterone	38,359	0
2-Thia-A-nor-5α-androstan-17β-ol	37,827	0.5
7α-Methyl-2-thia-A-nor-5α-androstan-17β-ol	37,080	2.5
7β-Methyl-2-thia-A-nor-5α-androstan-17β-ol	37,373	1.7
5α-Androst-2-en-17β-ol	37,209	2.1
2-Oxo-A-nor-5α-androstan-17β-ol	36,724	3.4
Anavar	38,052	0
Halotestin	37,815	0.6
Nilevar	36,798	3.2
Winstrol	37,363	1.7
5α-DHT (14×10^{-6} M)	37,850	0.5

Table 10. Effect of nonradioactive steroids on retention of radioactivity by ventral prostate *in vivo*.

^{3}H-5α-Dihydrotestosterone with nonradioactive steroids	Animal No.	Retention of radioactivity cpm/100mg	ave.	Inhibition of radioactivity uptake (%)
Control	1	3149		
	2	2724	2937	
	3	2937		
5α-Dihydrotestosterone	4	329		
	5	387	364	87.6
	6	375		
5α-Dihydrotestosterone 17-acetate	7	371	471	84.0
	8	570		
7β-Methyl-2-thia-A-nor-5α-androstan-17β-ol	9	1824	1561	46.9
	10	1297		
7β-Methyl-2-thia-A-nor-5α-androstan-17β-ol acetate	11	1643	1643	44.1
	12[a]	-		

a. Died after injection

(B) Results and discussion.

Following the incubation of minces from the rat ventral prostate with ^{3}H-DHT in Eagle's minimum essential medium, at 37° for 2 hr, a rapid accumulation of radioactivity appeared in the ventral prostate specimens. As shown in Table 5, the retention of radioactivity in ventral prostate of intact rats was not significantly different from that of castrate rats (one-day or three-day castration). It has been found (78,206) that removal of the main endogenous source of testosterone by castration of rats has a marked effect on the uptake and distribution of this hormone in some of the tissues. Although there is a significantly high uptake in some of the metabolic and excretory tissues like liver and kidney after castration, yet the uptake in target tissues for testosterone viz. prostate and seminal vesicle is not very different from that in intact animals. These earlier findings are well in accord with the present study. However, the addition of excess amount of nonradioactive DHT to the incubation medium reduced the retention of radioactivity much more in the ventral prostate of castrate rats (ca 33%) than in that of intact rats (6%). This could be attributed to the endogenous DHT present in the prostate of intact rats. On this basis, it is suggested that the use of castrate animals is essential in the competition binding study. The advisability of using animals castrated 24 hr before also has been convincingly shown by Mangan et al. (207).

The retention of radioactivity in the ventral prostate of individual animals has been found to vary from 6,000 to 10,000 dpm/mg dried tissue after incubation with ^{3}H-testosterone for 2 hr (98). In order to avoid such individual variation as much as possible, in the present study 4 to 5 rats were used in each experiment and minces of ventral prostate were mixed thoroughly before dividing into portions. As shown in Table 5, when the incubations were carried out for 30 min, the retention of radioactivity in six experiments

varied from 205 to 294 cpm/mg wet tissue. The average retention of radioactivity was 263 cpm/mg. The addition of nonradioactive DHT to the incubation medium reduced the retention of radioactivity to from 131 to 187 cpm/mg. The average percent of inhibition of radioactivity uptake was 35%. These values obtained from 30-min incubation are essentially similar to those from 2-hr incubation.

Many synthetic steroids also competed with DHT for receptor binding. For example, Anavar (208), Halotestin (209,210), Nilevar (211-214), Winstrol (215-217), 5α-androst-2-en-17β-ol (65,218), and 2-thia-A-nor-5α-androstan-17β-ol (176) are weaker androgens than DHT, but almost all of them showed greater inhibitory effects than DHT for retention of radioactivity (Table 6). If the binding affinity of a steroid to receptor protein plays the sole role in determining the potency of the androgen, a compound which shows greater binding affinity should give greater androgenic activity. However, this is not the case for those steroids mentioned above because they showed similar receptor binding affinities, although their relative androgenicities are widely separated. Other factors, such as intrinsic activity, absorption, distribution, and metabolism can affect the androgenicities of these compounds. Therefore, although most structure-function theories concerning androgens and anabolic agents (as described in Part II) consider only drug-receptor affinity, it is obvious that this represents a gross oversimplification of the problem, and one that is no doubt responsible for the wide variation in the theories thus far advanced. On the other hand, the effects of C-7 methyl group on the androgenic and anabolic activities of the parent compound are obviously mediated through direct or indirect interaction of the methyl group with the receptor since in each series of testosterone, DHT, 19-nortestosterone, 19-nor-DHT, and 2-thia-A-nor steroid the introduction of the 7α-methyl group increases both androgenic and anabolic potencies of the parent compound

whereas the 7β-methyl group decreases both activities to a very large degree.

As shown in Table 6, 7β-Me-DHT, which is biologically less active than DHT, gave a lower inhibitory effect for radioactivity uptake, and 7α-Me-DHT, which is much more potent than DHT, showed an even lower inhibitory effect. In the 2-thia-A-nor steroid series, the inhibitory effects were roughly in the order of 7α-Me-thia > thia > 7β-Me-thia. Essentially, similar results have been reported by Liao et al. (70). As shown in Table 11, the introduction of 7α-Me group into testosterone, 19-nortestosterone, 19-nor-DHT, and their 17α-Me derivatives enhanced the abilities of all parent compounds to bind to β protein (cytoplasmic receptor for androgens). However, 7α-Me decreased the binding affinity of DHT while enhancing its androgenic activity. In the case of 7β-Me substituted steroids, the 7β-Me group decreased the binding affinity of parent compound to a very large degree.

The above findings strongly indicate that both receptor binding affinity and intrinsic activity must be involved in determining the potency of 7-Me substituted androgens. It is postulated that both β-face and thickness (between α-face and β-face) of steroid molecule play an important role in determining the intrinsic activity and the binding affinity. The binding affinity obtained from the competition experiment represent the number of molecules bound to the receptor protein. The intrinsic activity represents the degree of optimum change of receptor protein conformation induced by interacting with steroid molecule that is necessary for a receptor protein to interact with "acceptor" molecules in the nuclei and to trigger the hormone action (219). The flatter the β-face, the greater the intrinsic activity and the binding affinity. If the thickness of the molecule is too large or too small, both would decrease the binding affinity. The effect of axial 7α-Me group would be to flatten the β-face toward the receptor surface thus enhancing the intrinsic activity as well as the binding affinity. This

Table 11. Relative androgenic activities (RA) and relative competition indices (RCI) of various androgens.[a]

Steroid	R.A.[b]	RCI[c] Receptor binding
Testosterone	0.4	<0.1
7α-Methyltestosterone	0.4[d]	0.2
7β-Methyltestosterone	0.1	<0.1
17α-Methyltestosterone	0.4	0.1
7α,17α-Dimethyltestosterone	0.6	0.2
7β,17α-Dimethyltestosterone	0.1	<0.1
5α-Dihydrotestosterone	1.0	1.0
7α-Methyl-5α-DHT	1.2	0.4
17α-Methyl-5α-DHT	0.8	1.1
7α,17α-Dimethyl-5α-DHT	1.5	0.6
7β,17α-Dimethyl-5α-DHT	0.0	<0.1
19-Nortestosterone	0.2	0.9
7α-Methyl-19-nortestosterone	2.6	2.6
17α-Methyl-19-nortestosterone	0.3	1.2
7α,17α-Dimethyl-19-nortestosterone	5.7	3.5
19-Nor-5α-DHT	0.1	0.5
7α-Methyl-19-nor-5α-DHT	0.3	0.6
7α,17α-Dimethyl-19-nor-5α-DHT	0.3	1.0

a. Taken from Ref. 70.

b. See Ref. 70 for detailed description.

c. The RCI values between 0.05 and 0.1 are shown as 0.1 and that below 0.05 are shown as 0.0.

d. Ref. 19 gave a greater androgenic activity.

flattening effect would account for the large increase of androgenic activity in all series of androgens (e.g. DHT, testosterone, 19-nor-DHT, 19-nortestosterone, and 2-thia-A-nor steroid). However, 7α-Me group also increases the thickness of the molecule which may either increase or decrease the binding affinity depending on the absence or presence of C-10 angular methyl group. Thus, in the DHT series 7α-Me group enhances the intrinsic activity as well as the binding affinity by flattening the β-face, but decreases the binding affinity by increasing the thickness of the molecule. The effect of enhanced intrinsic activity would overcome the effect of decreased binding affinity, so that the net effect is the increase of androgenic activity although the net binding affinity decreased. On the other hand, remonal of C-10 angular methyl group from DHT (e.g. 19-nor-DHT) decreases the thickness of the molecule thus decreasing the binding affinity. However, the introduction of 7α-Me group into 19-nor-DHT series restores the thickness of the molecule which, coupled with the flattening effect, increases the binding affinity as well as the intrinsic activity. In 2-thia-A-nor steroid series because of the presence of relatively bulky sulfur atom in the A ring, the flattening effect by 7α-Me group would probably be greater than that in DHT series, and the 7α-Me group would not increase the thickness of the molecule very much. Therefore, the net effect is the increase of androgenic activity without changing the binding affinity. In the case of testosterone, the A ring of the molecule is rather bent downward which may account for the large decrease of binding affinity compared with DHT. The introduction of 7α-Me group increases the binding affinity by the flattening effect but would not increase the thickness of the molecule, so that the net binding affinity increased. The 9α-F in Halotestin has a more prominent effect on the binding affinity presumably by the same mechanism operating in testosterone. The effect of 7α-Me group on the binding affinity and the intrinsic activity is

much greater in the 19-nortestosterone series than in any other series mentioned above. Like testosterone, the A ring of 19-nortestosterone is also bent downward, but due to lack of C-10 angular methyl group, the thickness of the molecule is smaller than that of testosterone although greater than that of 19-nor-DHT (probably similar to that of DHT). Thus, the binding affinity of 19-nortestosterone (as well as Nilevar) is comparable to that of DHT. The introduction of the 7α-Me group not only increases the binding affinity by increasing the thickness of the molecule, but also increases both the intrinsic activity and the binding affinity by the flattening effect. This reinforced effect would account for the large increases of the binding affinity as well as the androgenic activity.

Since the 7β-methyl group is in the plane of steroid molecule, the direct interaction of this substituent with the third-dimension of the receptor protein would account for the decreased binding affinity. However, in view of the nonandrogenic action of 7β-methyl-2-thia-A-nor steroid which still showed receptor binding affinity, the major effect of 7β-methyl group must be to decrease the intrinsic activity, thus reducing the androgenic activity. This view is further supported by the finding that cyproterone, a potent antiandrogen, showed an inhibitory effect essentially comparable to that of DHT whether the incubations were carried out for 30 min (Table 6) or 2 hr (Table 7). Due to the similarity between 7β-methyl-2-thia-A-nor steroid and cyproterone in binding affinity-intrinsic activity, one would expect that 7β-methyl-2-thia-A-nor steroid would be a potent antiandrogen.

A difficulty in the interpretation of these data is posed by the fact that when the incubations were carried out for 2 hr as shown in Table 7, the binding affinities of 7-methyl substituted DHT were clearly in the order of 7α-methyl-DHT (48-51%) >DHT (29-32%) > 7β-methyl-DHT (17%). These data suggest that the increased androgenic activity of 7α-methyl-DHT is due mainly

to the increased binding affinity in contrast to the results described above. In these incubations, which were carried out in air, the pH value of the medium was over 9.5 at the end of incubation period. At this pH value the receptor proteins might undergo conformational changes favoring the binding of 7α-methyl-DHT. Since the 30-minute incubations gave results similar to those obtained using cytosol fraction (70), the values obtained from the two-hour incubations are probably different only because of the change in pH. This point could be clarified by controlling the pH of the longer incubations.

In the competition experiments an excess of nonradioactive steroids must be added to the incubation medium in order to compare their binding affinities to receptor protein. Because of the poor solubility of steroids in water and the similar structure of most steroids, if the amount of nonradioactive steroid added to the incubation medium is beyond its solubility limit, it may cause precipitation of the radioactive steroid leading to a false inhibitory effect of such a steroid for the retention of radioactivity by tissue specimen. For example, as shown in Table 8, DHT and 2-thia-A-nor steroid at a concentration of 25 μg/ml precipitated 25% and 20% of ^{3}H-DHT respectively. Other steroids such as 5α-androst-2-en-17β-ol and 5α-androstan-17β-ol also caused significant precipitation of ^{3}H-DHT. However, as shown in Table 9, all steroids used in the present study at a concentration of 3.4×10^{-6} M did not precipitate ^{3}H-DHT. This indicates that inhibitions of radioactivity uptake obtained in the present study are results of competition for receptor binding, and not of precipitation of ^{3}H-DHT caused by the addition of nonradioactive competitors.

As shown in Table 7, the binding affinity of DHT was greater than that of DHT 17-acetate. Similarly, the 7β-methyl-2-thia-A-nor steroid was strongly bound, whereas its acetate was devoid of binding affinity. This indicates that the 17β-hydroxy group is important in the binding of steroid

to the receptor protein. In the *in vivo* competition experiments, as shown in Table 10, both DHT and its acetate showed a comparable inhibitory effect for the retention of radioactivity. The 7β-methyl-2-thia-A-nor steroid and its acetate also showed a similar inhibitory effect. These data strongly suggest that the nonandrogenic action of 7β-methyl-2-thia-A-nor-5α-androstan-17β-ol is due mainly to lack of intrinsic activity. These findings further support the view pointed out earlier by Wolff and Jen (60) that the lack of androgenic activity does not necessarily mean a lack of interaction with the receptor and the conclusion that the effect of 7-methyl group on the biological activity of parent compound is mediated at the receptor affinity-intrinsic activity level.

Although highly heterogeneous preparations were used to carry out the steroid receptor binding experiments, the results of the present study are well in line with those using homogeneous fractionated target organ preparations (70). In general, the relationship between binding affinity and chemical structure could be studied by the *in vitro* incubation experiments.

PART VI

ANTAGONISTIC ACTION OF ANTIANDROGENS AND DISSOCIATION OF ANDROGEN RECEPTORS

(A) Anti-androgenic anabolic tests (22,191)

Sprague-Dawley male rats 21 days of age were used for experiments the day after castration. The test compounds in CMC solution were given by subcutaneous administration (each compound in a separate solution at a separate site) once daily for seven days to the rats at the start of the test. The 2-thia-A-nor steroid was given 20 min before DHT. Preliminary experiment determined the optimally equivalent androgenic doses to be 1 mg of DHT and 3 mg of Δ^2 (total dose).

(B) Results

As shown in Tables 2 and 12, DHT and Δ^2 in ratio of 1:3 showed about equivalent activity in androgenic-anabolic tests. Cyproterone reduced almost equally the androgenic responses of ventral prostate and seminal vesicles to DHT and Δ^2. It also reduced the levator ani response to DHT but had no effect on that to Δ^2. These results strongly indicate a qualitative difference in the actions of DHT and Δ^2 on the levator ani. The data also support the view that Δ^2 stimulates primarily anabolic receptor of the levator ani muscle, while DHT stimulates both androgenic and anabolic receptors. On the other hand, Δ^2 and DHT stimulate the same receptors of ventral prostate and seminal vesicle responsible for androgenic activity.

Like cyproterone, 7β-methyl-2-thia-A-nor-5α-androstan-17β-ol, which is devoid of androgenic activity (see Table 2), largely inhibited the uptake of ^{3}H-DHT by ventral prsotate *in vivo* (Table 10) and *in vitro* (Table 6). However, in the anti-androgenic anabolic tests as shown in Tables 13 and 14,

Table 12. Anti-androgenic anabolic tests

Treatment Total Dose		Body Weight Initial (gm)	 Final (gm)	Ventral Prostate (mg)	Seminal Vesicles (mg)	Levator Ani (mg)
Controls		54	95	15.8	12.0	25.9
CMC		58	94	18.8	12.2	25.4
		56	93	16.1	9.7	24.0
		53	89	16.3	12.0	23.6
		56	90	18.9	12.4	21.8
	Ave.	55	92	17.2	11.7	24.1
	S.E.±	0.87	1.16	0.69	0.50	0.72
Cyproterone		56	90	24.5	14.2	29.0
16 mg		55	92	24.6	15.6	22.3
+		54	85	20.0	15.6	19.9
CMC		55	88	27.3	12.8	23.3
		56	85	24.6	15.8	20.2
	Ave.	55	88	24.2	14.8	22.9
	S.E.±	0.37	1.38	1.18	0.58	1.64
DHT		56	94	120.7	48.9	58.5
1 mg		53	77	116.2	60.3	58.6
+		54	98	149.2	62.6	73.8
CMC		56	95	140.3	71.4	58.3
		57	107	105.9	54.3	77.5
	Ave.	55	94	126.5	59.5	65.3
	S.E.±	0.73	4.87	7.97	3.82	4.25
DHT		56	85	80.2	37.6	44.2
1 mg		56	89	100.6	43.0	41.4
+		54	89	86.7	35.6	49.8
Cyproterone		56	91	102.6	45.4	43.8
16 mg		55	81	92.7	40.9	45.0
	Ave.	55	87	92.6	40.5	44.8
	S.E.±	0.40	1.79	4.20	1.77	1.38
5α-Androst-2-en-17β-ol		58	110	118.8	70.3	71.5
3 mg		54	88	104.7	63.8	50.8
+		55	98	118.5	65.4	64.0
CMC		56	97	104.4	66.4	74.4
		55	93	103.4	65.6	71.8
	Ave.	56	97	110.0	66.3	66.5
	S.E.±	0.68	3.65	3.55	1.09	4.29

Table 12. (continued)

Treatment Total Dose		Body Weight Initial (gm)	Body Weight Final (gm)	Ventral Prostate (mg)	Seminal Vesicles (mg)	Levator Ani (mg)
5α-Androst-2-en-17β-ol		55	91	58.8	38.4	65.6
3 mg		56	97	72.6	37.6	55.5
+		54	96	75.9	40.3	62.8
Cyproterone		58	98	76.2	37.6	62.6
16 mg		55	88	76.2	50.6	63.5
	Ave.	56	94	71.9	40.9	62.0
	S.E.±	0.68	1.92	3.35	2.47	1.71

Table 13. Anti-androgenic anabolic tests

Treatment Total Dose		Body Weight Initial (gm)	Body Weight Final (gm)	Ventral Prostate (mg)	Seminal Vesicles (mg)	Levator Ani (mg)
Controls		67	105	18.6	16.3	30.0
CMC		63	93	21.4	14.8	30.5
		65	95	20.3	14.7	32.8
		61	100	22.8	13.6	28.0
		65	98	24.4	14.4	30.8
	Ave.	64	98	21.5	14.8	30.4
	S.E.±	1.02	2.08	0.10	0.44	0.77
DHT		60	98	126.0	62.3	54.2
1 mg		65	101	130.7	72.6	75.6
		63	68(a)	139.6	74.0	56.2
		68	100	102.3	63.2	56.7
		63	96	139.7	69.0	67.2
	Ave.	64	93	127.7	68.2	62.0
	S.E.±	1.32	6.21	6.87	2.38	4.09
DHT		60	96	110.8	60.0	68.4
1 mg		64	100	126.3	66.2	59.2
+		65	104	110.2	69.0	70.6
7β-Me-2-thia-A-nor-		62	104	117.6	70.5	72.2
5α-androstan-17β-ol		65	90	152.4	74.0	68.2
acetate						
6 mg	Ave.	63	99	123.5	67.9	67.7
	S.E.±	0.97	2.65	7.80	2.35	2.25

a. Lung infection

Table 14. Anti-androgenic anabolic tests

Treatment Total Dose		Body Weight Initial (gm)	 Final (gm)	Ventral Prostate (mg)	Seminal Vesicles (mg)	Levator Ani (mg)
Controls		56	85	19.5	13.0	24.4
CMC		54	86	24.8	16.2	20.0
		56	90	21.5	15.9	22.9
		55	89	21.0	15.2	21.8
		58	85	22.3	11.3	25.0
	Ave.	56	87	21.8	14.3	22.8
	S.E.±			0.87	0.94	0.90
DHT		55	91	112.8	45.2	62.7
0.5 mg		54	91	93.2	40.1	50.3
		56	95	67.8	42.3	55.6
		56	95	87.2	33.8	54.3
		58	91	73.2	31.9	54.2
	Ave.	56	93	86.8	38.7	55.4
	S.E.±			7.95	2.52	2.02
7β-Me-2-thia-A-nor-		58	91	111.3	50.0	51.1
5α-androstan-17β-ol		56	92	107.3	51.1	56.9
acetate		55	84	92.7	51.9	54.2
25 mg		56	94	70.4	39.9	56.2
+		54	85	97.5	45.0	50.6
DHT	Ave.	56	89	95.8	47.6	53.8
0.5 mg	S.E.±			7.18	2.26	1.29

this compound did not antagonize the androgenic action of DHT on the ventral prostate and the seminal vesicles.

(C) Discussion

Dissociation of androgenic and anabolic properties of steroids by anti-androgenic and anti-anabolic agents in rats has been reported by other workers (152). It was postulated that the levator ani muscle may have 2 sets of receptors, one for androgenic and one for anabolic activity. Stimulation of either type of receptor leads to muscle growth. The ventral prostate and the seminal vesicle seem only to have androgenic receptors. This earlier suggestion is well in line with the present study. The 2-thia-A-nor steroid, although inhibiting the uptake of ^{3}H-DHT by ventral prostate in a short time *in vivo* and *in vitro* experiments, may have a very short half-life and for this reason it may not be able to antagonize the androgenic action of DHT in the anti-androgenic anabolic tests. It is also possible, though less likely, that a relatively large amount of 2-thia-A-nor steroid might show a certain degree of androgenic activity under such experimental condition.

In view of the antagonistic action of cyproterone on the responses of the ventral prostate and the seminal vesicles to DHT and Δ^2, it is of special interest in connection with the earlier findings (130) that the *in vitro* binding of the radioactive Δ^2 to minced rabbit prostate was not inhibited by DHT and cyproterone and that there are different binding sites in the ventral prostate for DHT and Δ^2. These earlier findings are summerized in Table 15. In addition to the inconsistency mentioned above, some others were observed. The earlier findings showed that Δ^2 did not inhibit ^{3}H-DHT uptake by intact rabbit prostate and the addition of double amount of nonradioactive steroid to the incubation medium inhibited about 60% of radioactivity uptake

Table 15. Effect of nonradioactive steroids on retention of radioactivity by minced rabbit ventral prostate.

Nonradioactive steroids	% of inhibition of radioactivity uptake		
	^{3}H-DHT	^{14}C-Δ^2	^{14}C-HC
17α-Me-5α-DHT	31.9	0	0
17α-Me-5α-androst-2-en-17β-ol (Δ^2)	0	57.8	0
17α-Me-5α-androstan-17β-ol (HC)	0	0	63.2
2-Thia-A-nor-5α-androstan-17β-ol	25.8	16.5	0
Cyproterone	35.9	0	0

a. Taken from Ref. 130.

b. Amounts of radioactive steroids: Δ^2 and HC, 25 μg/ml; DHT, 1.25×10^{-3} μg/ml.

c. Amounts of nonradioactive steroids: 50 μg/ ml.

d. Minced ventral prostate from intact rabbit were suspended in 4 ml of Krebs-Ringer phosphate buffer (pH 7.4) containing steroids and incubated under an atmosphere of air for 2 hr at 37° in a shaking bath.

e. Results are mean values of four determinations.

f. The specific activities of ^{14}C-Δ^2 and ^{14}C-HC were 99.5 μCi/mMole and 90.9 μCi/mMole, respectively.

Table 16. Solubility of steroids in Krebs-Ringer phosphate buffer (pH 7.4).

Steroids	Precipitation observed	Radioactivity in filtrate cpm/ml	Retention of radioactivity in filtrate (%)
17α-^{14}C-Methyl-5α-androst-2-en-17β-ol (25 μg/ml)			
in EtOH	-	4,370	
in buffer solution	+	806	18.4
17α-^{14}C-Methyl-5α-androstan-17β-ol (25 μg/ml)			
in EtOH	-	3,200	
in buffer solution	+	89	2.8
^{3}H-5α-Dihydrotestosterone (1.67×10^{-3} μg/ml)			
in EtOH	-	211,000	
in buffer solution	+	214,000	100
with 17α-methyl-5α-DHT (50 μg/ml)	+	71,000	23.6
with 2-thia-A-nor steroid (50 μg/ml)	+	116,000	55.0

a. The buffer solution containing 2.5% EtOH was used.

b. 17α-^{14}C-Methyl-5α-androst-2-en-17β-ol and 17α-^{14}C-methyl-5α-androstan-17β-ol were prepared by Dr. Yasuji Kasuya (see Table 15).

Table 17. Effect of nonradioactive steroids on retention of radioactivity by minces of rabbit ventral prostate.

Nonradioactive steroids (25 μg/ml)	Inhibition of radioactivity uptake (%) ^{3}H-HC Exp. 1	^{3}H-HC Exp. 2	^{3}H-Δ^2 Exp. 3
5α-Androstan-17β-ol (HC)	62.7	62.4	64.1
5α-Androst-2-en-17β-ol (Δ^2)	57.7	53.6	61.8
2-Thia-A-nor-5α-androstan-17β-ol	44.0	38.9	40.8

a. Results are mean values of four determinations.

b. ^{3}H-HC (5.0 mCi/mMole) and ^{3}H-Δ^2 (3.3 mCi/mMole) were prepared by Dr. Yasuji Kasuya and purified by thin layer chromatography.

c. Concentration of radioactive steroids used was 6.25 μg/ml.

d. In each experiment two intact rabbits were used.

e. See Ref. 130 for experimental procedure.

Table 18. Effect of HC and Δ^2 on retention of ^{3}H-HC by minces of rabbit ventral prostate.

^{3}H-HC (6.25 μg/ml) with nonradioactive steroid	In minces of prostate Retention of radioactivity cpm/mg	Inhibition of radioactivity (%)	In buffer solution Radioactivity remained cpm/ml	Inhibition of radioactivity(%)
None (control)	70.9		7,150	
HC (25 μg/ml)	25.0	64.7	3,220	55.0
Δ^2 (25 μg/ml)	36.3	48.8	3,400	52.4

a. Results are mean values of two determinations.

b. Two intact rabbit were used.

c. See Ref. 130 for experimental procedure.

Table 19. Effect of 5α-DHT on retention of ^{3}H-5α-DHT by minces of rabbit ventral prostate.

^{3}H-5α-DHT (0.25 μg/ml) with 5α-DHT (25 μg/ml)	Sample No.	Retention of radioactivity cpm/mg	ave.	Inhibition of radioactivity (%)
Group 1 (30-min incubation)				
Control	1	74.5	73.6	
	2	72.6		
5α-DHT added	1	53.8	54.2	26.4
	2	54.5		
Group 2 (60-min incubation)				
Control	1	142.2	138.7	
	2	135.2		
5α-DHT added	1	105.9	103.8	25.2
	2	101.7		
Group 3 (without tissue)				25.2

a. Two intact rabbits were used.

b. See Ref. 130 for experimental procedure.

by intact rabbit prostate. However, the present study showed that Δ^2 did inhibit ^{3}H-DHT uptake by castrate rat ventral prostate and its inhibitory effect was comparable to that of DHT (see Table 6). In addition, as shown in Table 5, the addition of more than 3,000 times as much nonradioactive DHT inhibited about 35% of radioactivity uptake by castrate rat prostate, whereas less than 10% inhibition was observed in the intact animals.

By examining the experimental conditions used by these workers, it was found that: (a) the amount of radioactive Δ^2 and HC (25 μg/ml) as well as nonradioactive steroids (50 μg/ml) used in the in vitro competition experiments are far beyond the solubility limit of steroid in the aqueous solution, (b) it is unlikely that the inhibition of radioactivity uptake by minces of intact rabbit prostate as large as 60% could be obtained upon addition of only double amounts of nonradioactive steroids, (c) it is also unlikely that such large inhibitions could be demonstrated in the prostate of intact animals. As discussed before, steroids have very limited solubility in the aqueous solution and due to very similar structure one can depress the solubility of another. When the amount of radioactive Δ^2 and HC used by those workers was tested for their solubilities in the incubation medium, as shown in Table 16, it was found that only 18.4% of Δ^2 and 2.8% of HC remained in the medium. One would expect further coprecipitation of radioactive steroid upon addition of nonradioactive steroid. Indeed, after the addition of 17α-methyl-DHT (50 μg/ml) and 2-thia-A-nor steroid (50 μg/ml) into the incubation medium containing ^{3}H-DHT (1.67×10^{-3} μg/ml), the radioactivities remaining in the medium were 33.6% and 55.0% respectively (Table 16). Thus, the precipitation of radioactive steroid caused by the use of large amount of radioactive steroids or the addition of large amount of nonradioactive steroids into the aqueous incubation medium could account for the large inhibition of radioactivity uptake by rabbit prostate (Table 15). Further

evidence was given in Tables 17, 18, and 19. As shown in Table 17, all HC, Δ^2, and 2-thia-A-nor steroid inhibited both radioactive HC and Δ^2 uptakes by the intact rabbit prostate. These are not in harmony with the earlier findings (130) which showed that HC did not inhibit radioactive Δ^2 uptake and that Δ^2 and 2-thia-A-nor steroid did not inhibit radioactive HC uptake. In fact, the inhibitory effect as shown in Table 17 was a result from the precipitation of radioactive steroid. In Tables 18 and 19, the correlation between the percent inhibition of radioactivity uptake by the intact rabbit prostate and the percent precipitation of radioactive steroid in the incubation medium was clearly indicated.

The above data in the present study strongly indicate that the earlier findings (130) regarding three separate binding sites in the ventral prostate for DHT, Δ^2, and HC were made from the incorrect interpretation of the inhibition of the radioactivity uptake by ventral prostate. Due to very low specific radioactivity of HC (5.0 mCi/mMole) and Δ^2 (3.3 mCi/mMole), it is very difficult to carry out competition study under the experimental conditions described in Part V. The best way to determine whether there are three separate binding sites in the ventral prostate would be the use of radioactive Δ^2 and HC with very high specific activity.

SUMMARY

Since elimination of androgenic activity is essential for the development of drugs useful in the treatment of female breast cancer, one approach has been to look for the separation of biological activities by chemical modification of the steroid molecule. The rationale for this lies in the idea that minor chemical alteration of the steroid molecule may selectively increase certain features of biological activity of the parent compound with concomitant reduction in undesirable activities.

In the present study, two compounds, namely 7β-methyl-5α-dihydrotestosterone and 7β-methyl-2-thia-A-nor-5α-androstan-17β-ol, were designed on the basis of findings that the introduction of the 7β-methyl group into testosterone (e.g. Calusterone) increases the antitumor activity, while decreasing both androgenic and anabolic activities, and that the enhancing groups known to be useful in the carbocyclic steroid series could be introduced into the heterocyclic steroid series to give similar activity.

The preparation of 7β-methyl-5α-dihydrotestosterone and its 2-thia-A-nor analog was studied. 6-Dehydro-7-methyltestosterone was chosen as a key intermediate and prepared in good yield by a reported procedure. Hydrogenation in glacial acetic acid in the presence of palladium on charcoal afforded 7β-methyl-5α-dihydrotestosterone, which had a positive CD curve and a positive Cotton effect in the ORD. On this basis it was assigned the 5α-configuration. The assignment of the 7β-configuration was made on the basis of the catalytic hydrogenation of the dienone system in the steroid molecule, which would be expected to proceed by cis-addition of hydrogen to the α-face, and the fact that the compound was not identical to 7α-methyl-5α-dihydrotestosterone. Cleavage of A ring with CrO_3-HOAc gave diacid which was converted to dibromide by the modified Hunsdiecker reaction. By cyclization in the presence of Na_2S, the dibromide gave 7β-methyl-2-thia-

A-nor-5α-androstan-17β-ol.

Biological evaluation showed that 7β-methyl-5α-dihydrotestosterone was only weakly androgenic, whereas 7β-methyl-2-thia-A-nor-5α-androstan-17β-ol was devoid of androgenic activity. These results are in good agreement with previous findings that 7α-methyl substitution increases both androgenic and anabolic potencies, whereas 7β-methyl substitution decreases both activities to a very large degree. From this, it was concluded that the introduction of the 7-methyl group into the steroid molecule affects drug-receptor interaction, and not drug distribution or drug metabolism. The effect of the methyl group on 7-position may be direct (in the case of 7β), by interaction with the receptor or indirect (in the case of 7α), by altering the conformation of the steroid itself through conformational transmission.

At the dose levels used both 7β-methyl-5α-dihydrotestosterone and its 2-thia-A-nor analog were non-toxic and produced no significant tumor-inhibitory effects. However, the final tumor size in animals treated with 7β-methyl-2-thia-A-nor steroid was significantly reduced. Since toxic effects were not evident and only one dose level was used, there is little question that larger doses could be safely tolerated. Based upon the limited results of this study, the determination of the tumor-inhibitory action of 7β-methyl-5α-dihydrotestosterone and its 2-thia-A-nor analog would have to be carried out at dose levels of about 50 mg/Kg/day.

The effect of nonradioactive steroids on retention of ^{3}H-5α-dihydrotestosterone by ventral prostate in vitro was studied. The data in the present study, coupled with previous findings, suggest that both receptor binding affinity and intrinsic activity are involved in determining the potency of 7-methyl substituted androgens and both β-face and thickness of steroid molecule play an important role in determining the intrinsic activity and the binding affinity.

Efforts were made to correlate chemical structure with receptor binding affinity and biological activity. The result from *in vivo* competition experiment strongly indicates that the nonandrogenic action of 7β-methyl-2-thia-A-nor-5α-androstan-17β-ol is due mainly to lack of intrinsic activity. Thus, like cyproterone, 7β-methyl-2-thia-A-nor-5α-androstan-17β-ol largely inhibited the uptake of ^{3}H-5α-dihydrotestosterone by ventral prostate *in vivo* and *in vitro*. However, in the anti-androgenic anabolic tests, this compound did not antagonize the androgenic action of 5α-dihydrotestosterone on the ventral prostate and the seminal vesicles. This may be attributed to the short half-life of the compound.

The previous findings regarding three separate binding sites in the ventral prostate for 5α-dihydrotestosterone, 17α-methyl-5α-androstan-17β-ol, and 17α-methyl-5α-androst-2-en-17β-ol was finally reinvestigated. Evidence strongly indicates that the previous findings were made from the incorrect interpretation of the inhibition of radioactivity uptake by ventral prostate. However, the question of the number of separate binding sites remains to be answered.

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