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Structure-activity relationships of androgens

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STRUCTURE-ACTIVITY RELATIONSHIPS OF ANDROGENS: METABOLISM AND TARGET ORGAN UPTAKE

by

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STRUCTURE-ACTIVITY RELATIONSHIPS OF ANDROGENS: METABOLISM AND TARGET ORGAN UPTAKE

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Abstract

The possibility that 5α-androstane derivatives having a simple hydrocarbon A ring can be oxygenated metabolically to known C-3 oxygenated androgens was examined. The following 3-oxygenated androstanes were isolated and characterized by glc and mass spectrometry after incubation of 17α-methyl-5α-androstan-17β-ol 12 with rabbit liver homogenate: 17β-hydroxy-17α-methyl-5α-androstan-3-one 15, 17α-methyl-5α-androstane-3α,17β-diol 16, and 17α-methyl-5α-androstane-3β,17β-diol 17. The results obtained from a study of the metabolism of 15, 16, and 17 by rabbit liver homogenate show that 15 and 16 largely give the corresponding derivatives 16 and 17, and 15 and 17, respectively, whereas only a small conversion of 17 to 15 and 16 was observed.

The *in vivo* study of uptake of 17α-^{14}C-methyl-5α-androstan-17β-ol ^{14}C-12 and 17α-^{14}C-methyl-5α-androst-2-en-17β-ol ^{14}C-14 by rabbit ventral prostate showed that the majority of the radioactivity recovered from this target organ was associated with the unchanged parent compounds after the administration of ^{14}C-12 and ^{14}C-14 orally or intramuscularly.

A study of the *in vitro* binding of the three radioactive androgens 1,2-^{3}H-5α-dihydrotestosterone ^{3}H-2, ^{14}C-12, and ^{14}C-14 to minced rabbit prostate was also carried out. The competitive effect of several other steroids on the binding of these labeled steroids seems to indicate the presence of three different binding sites for such compounds: The "classical" dihydrotestosterone site, as well as separate sites for the A ring hydrocarbon and the 2-olefin derivatives. The implications of this for structure-activity relationships of androgen analogs have been discussed.

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This research was supported in part by Public Health Service Grants (AM-05016 and AM-14824) from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service.

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INTRODUCTION

There have been exciting developments, especially in the past five to seven years, in our knowledge of the interaction of male sex hormones with cellular constituents in target tissues. There is growing evidence that steroid hormone action at the target site is initiated by the association of the hormone with a specific protein receptor in the cytoplasm of the target tissue cell and the complex formed is subsequently transferred to specific nuclear sites. There have also been a number of experimental approaches to the question of the relationship of metabolic alterations of androgens to the nature of "active forms" of testosterone and other androgens in their target tissues.

Metabolism, and target organ uptake and retention of androgen analogs in relation to their action and the problem of primary hormone receptors are the subjects of this thesis. Specifically, the present study is concerned with i) the C-3 oxygenation of 17α-methyl-5α-androstan-17β-ol by rabbit liver homogenate, ii) the metabolism of 3-oxygenated 17α-methyl-5α-androstan-17β-ols on incubation by rabbit liver homogenate, iii) the *in vivo* uptake and metabolism of 17α-^{14}C-methyl-5α-androstan-17β-ol and 17α-^{14}C-methyl-5α-androst-2-en-17β-ol by rabbit ventral prostate, and iv) the *in vitro* uptake and retention of 17α-^{14}C-methyl-5α-androstan-17β-ol, 17α-^{14}C-methyl-5α-androst-2-en-17β-ol, and 17β-hydroxy-1,2-^{3}H-5α-androstan-3-one by rabbit ventral prostate.

PART I: C-3 OXYGENATION OF 17α-METHYL-5α-ANDROSTAN-17β-OL AND INTERCONVERSION OF 3-OXYGENATED 17α-METHYL- 5α-ANDROSTAN-17β-OLS BY RABBIT LIVER HOMOGENATE

A. INTRODUCTION

In our laboratory, an attemt has been made to evaluate the role of the 3-keto group in androgenic steroids in producing the biological response (1a-k). It has been concluded that the important factor in inducing the biological response is only the steric effect of the C-3 carbonyl group rather than its electronic characteristics (1i).

It is known, however, that steroids having an unsubstituted A ring, such as androstan-17β-ol (2,3,4) and 5α-androstane (5,6,7) are androgens. Kochakian (8) has reported the androgenic activity of 17α-methyl-5α-androstan-17β-ol in castrated mice after the oral administration of this compound. Nutting et al. (9) also showed that 17α-methyl-5α-androstan-17β-ol has significant oral anabolic and androgenic activity and this has been confirmed in the clinic (10). Dorfman and Kincl (11) have reported the relative androgenic activity of certain 3-deoxy C_{19} steroids which possess androgenic activities in some instances higher than that found for testosterone. These studies have led to the suggestion that androgenic activity is not dependent on the presence of oxygen substituents at C-3, although most of the steroids with high activity do have 3-keto or 3-hydroxyl groups.

Recently, Okada et al. (12) discovered the metabolic conversion by rabbit liver slices of the progestational 3-deoxy steroid 17α-ethyl-estr-4-en-17β-ol to 17α-ethyl-19-nor-testosterone. This observation prompted us to investigate the metabolic fate of 3-deoxy androstane derivative 17α-methyl-5α-androstan-17β-ol in rabbit liver homogenate to examine the possibility that ring A hydrocarbons, such as androstan-17β-ol, 17α-methyl-5α-androstan-17β-ol, or 5α-androstane, owe their activity to their conversion in vivo to compounds oxygenated at C-3.

B. GENERAL CONSIDERATIONS

i. Structure-Activity Relationships of Androgens

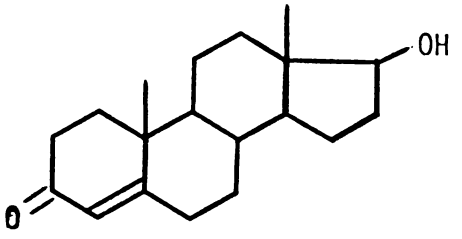
It has long been known that particular chemical structures produce particular biological actions. In the field of steroids, many structurally different steroids have been investigated in order to define the functional relationship between chemical constitution and physiological action.

Chemical structure-androgenic activity relationships have also been the subjects of extensive studies and are well documented in books (13,14, 15) and a recent review article (16). The main object of these structural studies has been to find chemical specificity or functional estimates of potency and affinity for the construction of theoretical models for androgen receptors. They are based on the principles of complimentariness, whereby the features of a molecule found to be important for potency have their mirror in the proposed model usually as dipoles, sites for hydrogen bond-

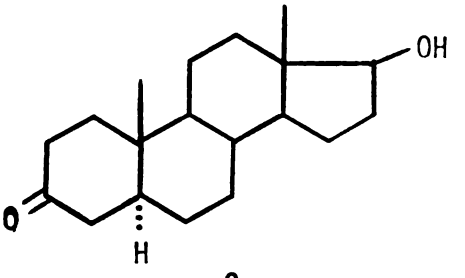
ing or hydrophobic areas laid out in a spatially critical array.

During the past two decades thousands of new steroids have been synthesized. Many of these are analogs of naturally occurring hormones. Some of these compounds have surpassed their basic prototype in biological activity; some have been less active or inactive, whereas others have exhibited types of hormonal activity not expected on the basis of their structures. However, it is true that within narrow and well defined boundaries certain unnatural substituents may be introduced on the basic steroid nucleus and in a fair percentage of cases an approximation of the biological activity of the resultant compound may be predicted in the experimental animal but not necessarily in man. From the steric conformation of these substituents and in some cases from their electronic contributions to key groupings on the steroid nucleus, certain preliminary generalizations concerning the relationship of structure and activity have been made.

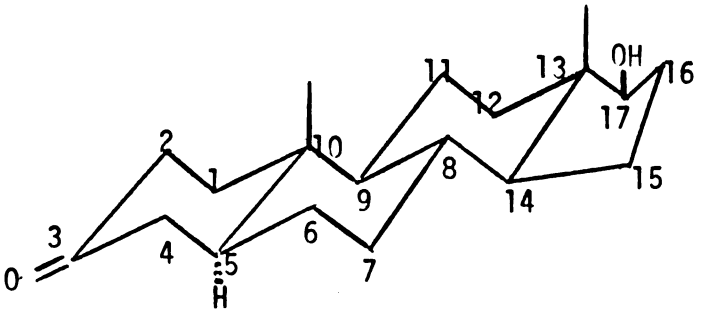
Ringold (13) has correlated the effects of alkyl groups and of electronegative group substitution with biological activity. The assumption was made that, at whatever site the steroid molecule is bound to elicit a specific biological activity, steric factors must be such that an intimate attachment is effected on only one face. As the prototypes of androgens, naturally occurring testosterone 1 and dihydrotestosterone 2 (17β-hydroxy-5α-androstan-3-one) have been selected. The author has come to the conclusion that classical androgenicity is dependent on an α-face (back side)

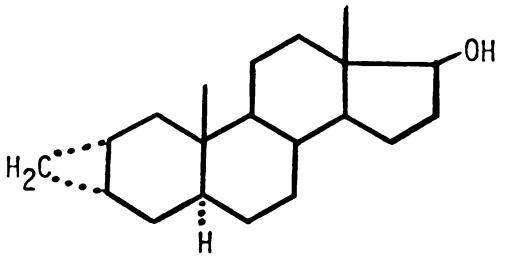


1
testosterone



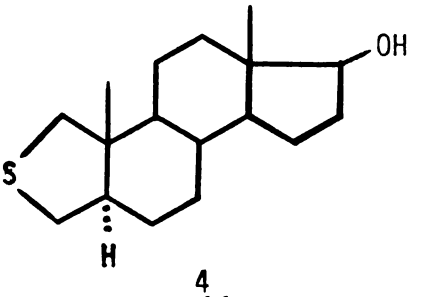
2
dihydrotestosterone
(17β-hydroxy-5α-androstan-3-one)





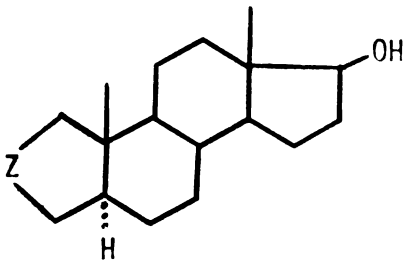
3

2α,3α-methano-5α-androstan-17β-ol



4

2-thia-A-nor-5α-androstan-17β-ol



5 Z = O
6 Z = Se
7 Z = Te
8 Z = S-S
9 Z = α-SO
10 Z = SO_2

interaction with the necessary cellular or coenzymatic surface on the basis of the observations of androgenic action of α- and β-alkyl- or halo-substituted androstanes.

Bush (17,18), however, has suggested that in the case of androgens the upper and β-sided surface of the molecule is responsible for conferring specificity on the steroid-receptor association. Bush (17) also suggested that the 17β-hydroxyl group plays a specific role in the association of this group with the androgenic receptors, since all active steroids in this group possess a 17β-hydroxyl group or else a group which can be converted to it. Furthermore, the author called attention 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.

Bowers et al. (19) have described the preparation of some Δ^1, Δ^2, Δ^3, and Δ^4 C-3 deoxyandrostanes and the importance of sp^2 hybridization at C-2 and C-3 in 17β-hydroxy androstanes for anabolic activity. Their basic concept was to vary the electron density pattern in and around ring A, since in conjunction with steric properties the electron density pattern should be related to the ability to bind to protein-like structures, for example, to charged subsites of enzymatic active centers. It was found that 1-, 2-, and 3-17β-hydroxy-5α-androstenes all exhibit reasonable anabolic activity, the anabolic-androgenic ratio being more favorable in the latter two compounds.

In contrast to the conclusion of Ringold (13), Wolff and Jen (20)

presented evidence for the hypothesis that androgens, as well as progesterone, function by β-face adsorption on the receptor site. In a later article (1a), Wolff and his co-workers described the preparation of a number of steroidal ring A olefins, epoxides, methano steroids, and spirooxiranyl steroids to verify their hypothesis (21) that the requirement for androgenic activity is the presence of high electron density at C-2 and/or C-3, such is provided by sp^2 hybridization. The hypothesis had been put forth independently from the similar hypothesis proposed by Bowers et al. (18) as described above. The biological evaluation of a number of these compounds by means of the myotrophic-androgenic assay indicated that the methano steroids, such as 2α,3α-methano-5α-androstan-17β-ol 3 are the most active compounds in the series. This observation resulted in the proposal that anabolic-androgenic androstanes are bound to their receptor by a β-face π bond to an sp^2 system in the A ring. To gain further information regarding the steric and electronic requirements in the A ring of anabolic-androgenic substances, the preparation and androgenic-myotrophic testing of analogs of 3 having substituents on the cyclopropyl ring have been undertaken (1d). If a π bond is important, the strength of the binding would be different in such analogs having altered electron density at the sp^2 centers. Since β-face binding was assumed to be involved, a substituent group on the cyclopropane ring of analogs of 3 should not interfere sterically with steroid-receptor binding. Two major conclusions have been drawn from this work. First, activity can

be related or enhanced in substituted compounds. This would not be expected on steric grounds if α-face adsorption were involved, but is in harmony with adsorption on the β-face of the steroid. Secondly, activity of a given analog is determined by the structure of the substituent on the cyclopropane ring.

The importance for androgenic activity of sp^2 atoms at C-2 and/or C-3 of steroids could be due to the steric characteristics of the sp^2 center, to the electronic nature of such atoms, or jointly, to both effects. The radicals -CH=CH- and -S- are considered isosteric. This is especially the case when cyclic derivatives are considered, and there are many examples of the similarity of the physical (22) and biological (23) properties of thiophene analogs of benzene compounds. Wolff and Zanati (1e) undertook the synthesis of an androstane analog in which sulfer replaced the sp^2 center at C-2, i.e., 2-thia-A-nor-5α-androstan-17β-ol 4 as an isostere of 5α-androst-2-en-17β-ol 5. The androgenic-anabolic activity of this compound was taken as evidence that steric effects, and not electronic factors, are important in connection with structural requirements at C-2 and/or C-3 in androgens. This finding has led them to examine the corresponding oxa- and selena-isologs (1i). The data from the pharmacological testing in this study indicate that selena- and thia-steroids 6 and 4 have comparable activity but oxa-, sulfoxide and sulfone derivatives 5, 9, and 10 are inactive. It has, therefore, been concluded that thia steroid 4 is almost

certainly active as such, and not as its most likely sulfoxide and sulfone metabolites 9 and 10. Furthermore, the covalent radii 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, it has been suggested that steric, and not electronic factors are involved in the C-2, C-3 structural requirements of androgens, based on the fact that selena-steroid 6 is active, whereas oxa-steroid 5 is inactive. Confirmation of this was provided by the activities of tellurio-steroid 7 which is somewhat more active than thia-steroid 4, and especially by disulfide 8, which is the most active compound in the series. Wolff (1i) concluded that an A ring equivalent in size to a six membered or larger carbocyclic ring, and having atoms which flatten the ring in the vicinity of C-2 and C-3, or their replacement, is important for androgenic activity. Electronic characteristics of the A ring are of minor importance. In related work, Zanati and Wolff (24) described the studies directed to the preparation of oxa-androstanes in which the oxygen atom was systematically moved around the A ring, since it is possible, in principle, to identify the exact area of the A ring where the flattening effect is required by the preparation of such derivatives. As would be expected if the nature of the effect of activity-engendering groups in the A ring is steric, the position of the O atom in the A ring is of crúcial importance to the biological activity. According to their results, the substitution of O in place of C-2, 2-oxa-5α-androstan-17β-ol, gives rise to the most active

compound. Substitution in place of C-3, 3-oxa-5α-androstan-17β-ol, results in a much less active compound, whereas substitution at C-4, 4-oxa-5α-androstan-17β-ol, results in a compound which is inactive. These results parallel those found by Bowers et al. (19) who "moved" a double bond around the A ring in a similar way. Since a double bond necessarily affects the geometry of 2 C atoms in the A ring, their data cannot be compared with those obtained by Zanati and Wolff (24). Nevertheless, the most active olefin is the Δ^2, whereas the least active is the Δ^4, so that the data, to the extent that it is possible to compare those of Bowers et al., are in general agreement. Bowers et al. (19) attributed their results primarily to changes in the electron density in the A ring, although they recognized that stereochemical differences might be important as well. Taken together, however, the data obtained by Zanati and Wolff (24) are clearly in strong support for the concept that the function of the structure of the activity-engendering group in ring A is wholly steric and that in principle isosteric groups of any type could be used to construct an androgenic molecule.

In connection with the concept that only the steric, and not the electronic, characteristics of ring A are important in eliciting androgenic action, Zanati and Wolff (25) described the synthesis of tricyclic compounds correspounding to steroids lacking ring A and they 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.

ii. Oxygen Function at C-3 and Androgenic Activity

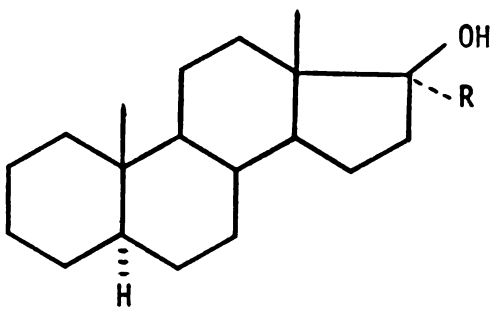
A basic structure and feature common to natural steroid hormones is the presence of oxygen function at C-3 of the ring A. Among the classical endocrine hormones the androgens are apparently unique in that an α,β-unsaturated ketone is not required for high hormonal activity and saturated 3-keto 5α-androstanes are potent agents as well as are the 3α-hydroxy androstanes. It has, however, been found that androgenic activity is not dependent on the presence of oxygen substituent at C-3, although the bulk of the steroids with high activity do have 3-keto or 3α-hydroxy groups. Active compounds which have no oxygen at C-3 are now known (Table 1). One of the earliest observations of this subject came from Kochakian (8), who reported that the oral administration of 17α-methyl-5α-androstan-17β-ol 12 did promote the grouwth of seminal vesicles and prostate to some extent and was relatively potent in anabolic effect in castrated mice. Huggins and Jensen (2) and Sydnor (3) found the androgenicity of 5α-androstan-17β-ol 11. Furthermore, Segaloff and Gabbard (5) and Segaloff (6) have demonstrated that 5α-androstane, possessing no oxygen function at C-3 and C-17 or any other carbon atom, has the ability to stimulate the seminal vesicles and prostate of the castrated rat as well as the growth of the chick comb. Dorfman et al. (7) have demonstrated that highly purified 5α-androstan-17β-ol 11 and 5α-androstane are 128% and 1.94% as androgenic as testosterone, respec-

Table 1. Relative Biological Activities of Steroids Related to 5α-Androstan-17β-ol (11) Having No Oxygen Function at C-3 of A ring (from Ref 16)

Steroids	Inunction		Injection		
			Rat		
	Chick Comb	Reference and (Standard)[a]	And.[b]	Anab.[b]	Reference and (Standard)[a]
5α-androstan	2	Dorfman et al. (6) (T)			
5α-androstan-17 -ol	128				
	---		35	100	Bowers et al. (19) (T)
	---		50	150	
	---		40	80	
	38	Dorfman & Kincl (4) (T)	10	10	
	4		---	---	
Δ^2_2 17α-CH_3	20		78	362	
Δ^2 2-CH_3	8		184	117	
Δ^2 2,17α-diCH_3	109	Dorfman & Kincl (4) (T)	76	310	Dorfman & Kincl (4) (T)
Δ^2 2-CH_2OH	1		---	---	
Δ^2_2 2-CH_2OH, 17α-CH_3	207		54	203	
Δ^2 2-CHO	<1		---	---	
Δ^2 2-CHO, 17α-CH_3	127		35	87	
Δ^2 2-CN, 17α-caproate	33		148	870	
2,3α-epithio			42	308	Klimstra et al. (26) (T)
2,3α-epithio, 17α-CH_3			27	154	
2,3β-epithio			2	13	
2,3β-epithio, 17α-CH_3			<1	<3	
2,3α-epoxy			<1	<1	
2,3α-epoxy, 17α-CH_3			1	5	
2,3β-epoxy			1	7	
2,3β-epoxy, 17α-CH_3			6	36	
2,3α-methano			30	100	Wolff et al. (1a) (TP)
2,3α-formylmethano			<100	>100	Wolff et al. (1d) (TP)

a Testosterone (T) or testosterone propionate as 100.

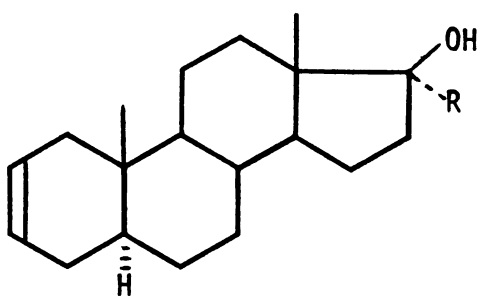
b And.: Androgenic; Anab.: Anabolic.



11

11 R = H, 5α-androstan-17β-ol

12 R = CH_3, 17α-methyl-5α-androstan-17β-ol



13 R = H, 5α-androst-2-en-17β-ol

14 R = CH_3, 17α-methyl-5α-androst-2-en-17β-ol

tively, in a chick's comb inunction assay. Since many other related steroids without a C-3 oxygen also have androgenic or anabolic activity, the presence of a 3-keto or 3-hydroxyl group mat not be absolutely necessary for hormonal activity. However, the possibility of an *in vivo* conversion of these steroids to 3-hydroxy steroids cannot be completely excluded. Especially, steroids with unsaturation at A ring, e.g., 5α-androst-2-en-17β-ol 13 may be hydroxylated in the animal.

iii. Absorption, Distribution and Metabolic Fate

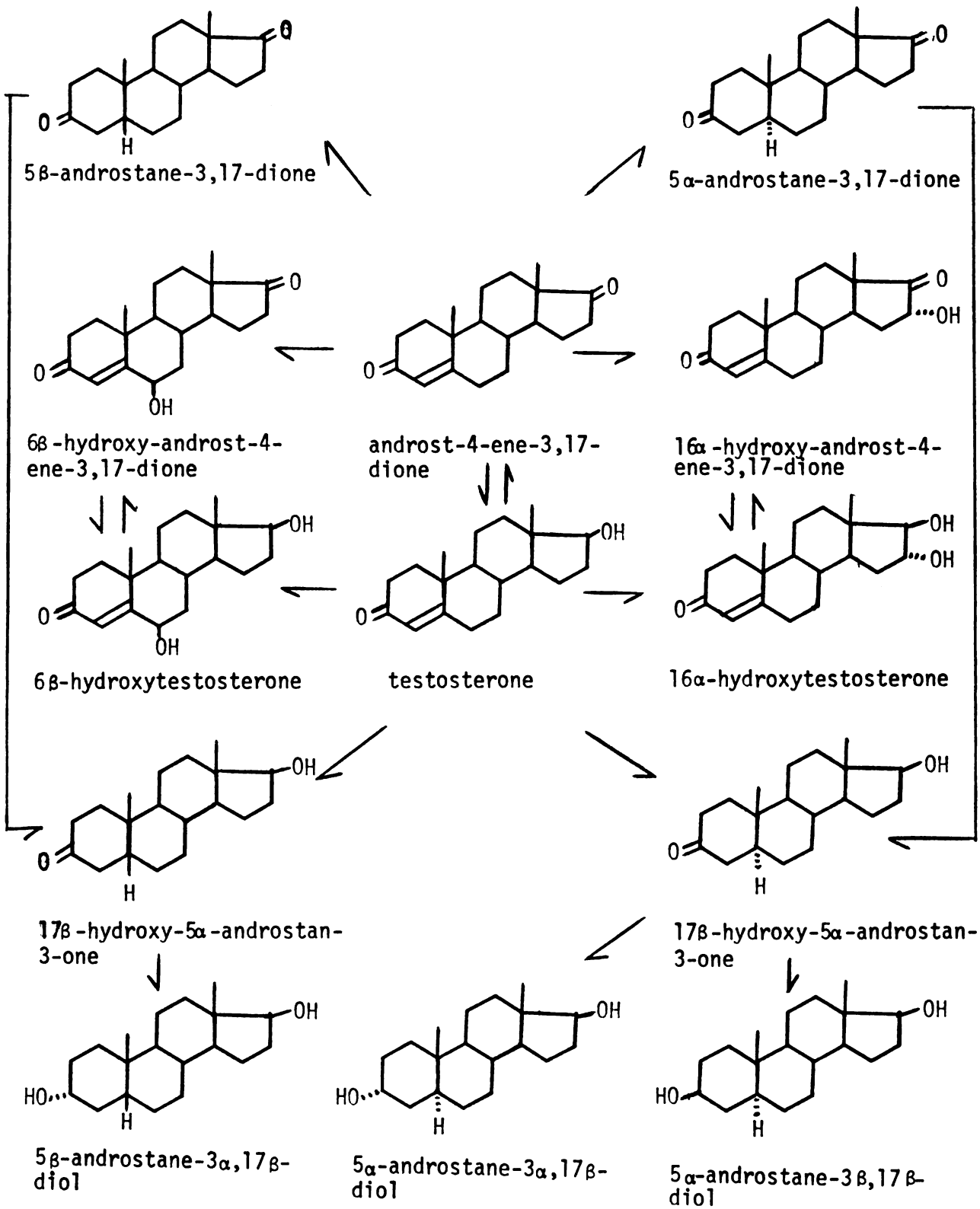
Although considerable research has been devoted to the synthesis of modified androgens, little is known about the absorption and distribution of these substances. It is well recognized that a steroid hormone might have intrinsic activity but exert little or no biological effects because its physicochemical characteristics prevent it from reaching the site of action. This is particularly true in humans, where slow oral absorption or rapid metabolic inactivation may greatly reduce the efficacy of the drug.

The present knowledge about the metabolism of testosterone is based mainly on *in vivo* investigations in man and *in vitro* studies with a variety of animal tissues. Samuels et al. (27) have found that testosterone, when incubated with human liver slices, is metabolized to 17-keto steroids. Later, Dorfman and his co-workers (28) identified a number of metabolites, including androst-4-ene-3,17-dione, androsta-4,16-dien-3-one, 5α-androstane-

3α,17β-diol, and 5β-androstane-3α,17β-diol after incubation of ^{14}C-testosterone with human liver homogenate. Fig 1 summarizes the results of the incubation experiments carried out by Lisboa et al. (29) using human liver slices. As can been seen, there exists in human liver a reductive as well as an oxidative pathway in the metabolism of testosterone. The reductive pathway leads to the formation of saturated 3,17-diols via the ring A saturated 3-keto-17β-hydroxy compounds; during the course of oxidative metabolism, both unsaturated and saturated 3,17-diketones as well as hydroxylated compounds are formed. Of particular interest is the fact that testosterone is reduced by human liver to the saturated diols, 5α-androstane-3α,17β-diol, 5α-androstane-3β,17β-diol, and 5β-androstane-3α,17β-diol. The fact that ring A saturated 3-keto-17β-hydroxy compounds are formed during incubation supports the view that the saturated diols arise from ring A saturated 3-keto steroids by reduction. These results are in agreement with the findings of Baulieu and Manvais-Jarvis (30) who made the important observation that, under in vivo conditions, testosterone is metabolized in man not only to 17-keto steroids but also to the saturated 5α- and 5β-androstanediols.

The metabolism of testosterone and other related compounds in various liver preparations has been studied extensively and numerous metabolites have been identified (31-38). It is well established that testosterone can be metabolized in various tissues other than the liver and those even include human skin by which testosterone was found to be actively metabolized

Fig. 1. Metabolism of Testosterone in Sices of Human Liver (from Ref 29)



under the influence of several enzymes, such as 17β-hydroxy oxidoreductase, 5α- and 5β-reductases (39,40).

A number of investigations have been carried out with target tissues, in particular the rat ventral prostate, to study the importance of testosterone metabolism in the target organ. Since some of the testosterone metabolites formed in the prostate exhibit known androgenic properties, it was necessary to find conditions for the simultaneous study of hormone metabolism and action in the same target organ, and to determine the correlation, if any, between them. These subjects will be discussed in greater detail in Part II.

Experimental Section

Steroids. 17α-Methyl-5α-androstan-17β-ol was obtained by Wolff-Kishner reduction (41) of 17α-methyl-5α-androstan-3-on-17β-ol. A mixture of 10 g of 17α-methyl-5α-androstan-3-on-17β-ol and 20 ml of hydrazine hydrate in 500 ml of ethylene glycol was maintained under reflux for 3 hr. Potassium hydroxide (10 g) was added and the internal temperature was raised to 190° by distillation and held there for 5 hr under reflux. On addition of 500 ml of water a precipitate formed which was extracted into ethyl acetate and washed to neutrality with water. The ethyl acetate extracts were dried over anhydrous sodium sulfate. Evaporation of the solvent left a residue which was recrystallized twice from CH_3OH to afford 4.8 g of 17α-methyl-5α-androstan-17β-ol, m.p. 154-7°.

17α-Methyl-5α-androstan-3-on-17β-ol (methylandrostanolone) was purchased from Searle Chemicals, Inc., Lot No. V-31.

17α-Methyl-5α-androstane-3β,17β-diol was made by reducing a solution of 17α-methyl-5α-androstan-3-on-17β-ol in CH_3OH with $NaBH_4$ (42). A solution of 3.12 g (10 mM) of 17α-methyl-5α-androstan-3-on-17β-ol in 75 ml of CH_3OH was added to a solution of 380 mg (10 mM) of $NaBH_4$ in 6 ml of H_2O and 30 ml of CH_3OH. The mixture warmed spontaneously and evolved gas. After standing at room temperature for 20 min, the solution was diluted with H_2O and extracted with ether. Recrystallization from acetone-petroleum ether yielded 2.6 g of white crystals. The crystals were recrystallized from acetone-petroleum ether again and 2.4 g of pure 17α-methyl-5α-androstane-

3β,17β-diol was obtained, m.p. 208-210°.

17α-Methyl-5α-androstane-3α,17β-diol was prepared by treatment of 5α-androstan-3α-ol-17-one in anhydrous benzene with CH_3MgBr (43). A solution of 5α-androstan-3α-ol-17-one (5 g) in anhydrous benzene (150 ml) was added with stirring to a 3 M solution of CH_3MgBr (Alfa Inorganics) in ether (100 ml). The solvent was removed until the boiling point reached 78° and refluxing was continued for 5 hr. After allowing the mixture to stand overnight at room temperature, a solution of NH_4Cl (15 g) in water (100 ml) was added dropwise with stirring. The layers were extracted alternately with $CHCl_3$ and ethyl acetate. The combined extract was washed with dilute HCl (1:3), 5% $NaHCO_3$ and H_2O. After drying the organic phase with anhydrous sodium sulfate, the solvent was removed by distillation. Recrystallization twice from ethyl acetate-petroleum ether gave 3.2 g of pure 17α-methyl-5α-androstane-3α,17β-diol, m.p. 180-2°.

All steroids used in incubations were examined for purity by thin-layer (tlc) and gas liquid chromatography (glc).

Preparation of Liver Homogenate and Incubation. Livers of male New Zealand white rabbits (4 kg) were weighed and homogenized in Waring blendor in Krebs-Ringer phosphate buffer (pH 7.4) at 4° (90 ml/10 g of tissue). The steroids were incubated with the liver homogenate for 15 min to 2 hr at 37° in a shaking bath, bubbling O_2 through the incubation mixture. Each incubation contained 100 ml of the liver homogenate (0.1 g of liver/ml)

and 4 mg of steroid in 1 ml C_2H_5OH. The enzymatic reaction was stopped at appropriate incubation time by the addition of 100 ml of CH_3OH-$CHCl_3$ (2:1, v/v) and, after shaking, the mixture was left in a refrigerator overnight.

Extraction. Coagulated proteins and precipitate were removed by filtration and washed with CH_3OH-$CHCl_3$ (2:1). The filtrate and washings were combined, concentrated under vacuum, and subjected to repeated extraction with petroleum ether (30^o-60^o). The petroleum ether extract was evaporated to dryness under vacuum, and the dried extract was acetylated with acetic anhydride and pyridine at 65^o for 2 hr. The mixture was evaporated and the residue was dissolved in a small amount of $CHCl_3$ and analyzed by gas chromatography.

Gas Chromatography and Mass Spectrometric Analysis. A Barber-Coleman Series 5000 gas chromatograph equipped with a flame ionization detector and a 1.8 m glass column was used. The glass column was packed with 3% OV-1 on 100-200 mesh Gas Chrom Q. Conditions employed were: column temperature 219^o; injector 240^o; detector 260^o; He 2.1 kg/cm^2; air 2.8 kg/cm^2; and H_2 1.4 kg/cm^2.

The vapors eluted from the column were condensed in capillary tubes. Compounds trapped from the effluents were subjected to mass spectrometry for final confirmation of identity.

Mass spectrometry was carried out with an AEI MS-902 high-resolution mass spectrometer (70 eV). For the mass spectrometric measurements the author thanks Dr. R. Weinkam.

Results

In the first part of this study, the ability of rabbit liver to oxygenate the position 3 of 17α-methyl-5α-androstan-17β-ol 12 to give rise to 3-keto 15, 3α-OH 16 and 3β-OH 17 was demonstrated.

Gas chromatography of the extract obtained from incubation with 12 indicates that the conversion of this compound to the 3-oxygenated androstanes occurs in the rabbit liver homogenate. Fig 2 shows the gas chromatographic data obtained after 60 minutes incubation. Peak 1 corresponds to the unchanged compound 12 and the metabolic transformation of this gives two distinctive peaks 2 and 3 before acetylation. Peak 2 has the same retention time as the 3-hydroxylated products of 17α-methyl-5α-androstan-17β-ol 12, i.e., 3α-OH 16 and 3β-OH 17 epimers. The retention time of peak 3 corresponds to 3-keto metabolite 15. The gas chromatographic behavior of these metabolites was examined by comparison with the authentic compounds synthesized.

Fig 3 clearly shows that the 3-OH epimers 16 and 17, which had the same retention time as shown by the dotted line, are separated after acetylation. That is, both 3α-AcO (peak 4) and 3β-AcO (peak 5) now have longer retention time than 3-keto 15 and the separation of these peaks is practical enough to collect pure samples for further identification of these metabolites by mass spectrometry.

Table 2 shows the mass spectrometric identification of the 3-oxygenated metabolites. Identical fragmentation patterns were obtained for

Fig. 2. Gas Chromatography: 3% OV-1 on Gas Chrom Q at 219° before Acetylation.

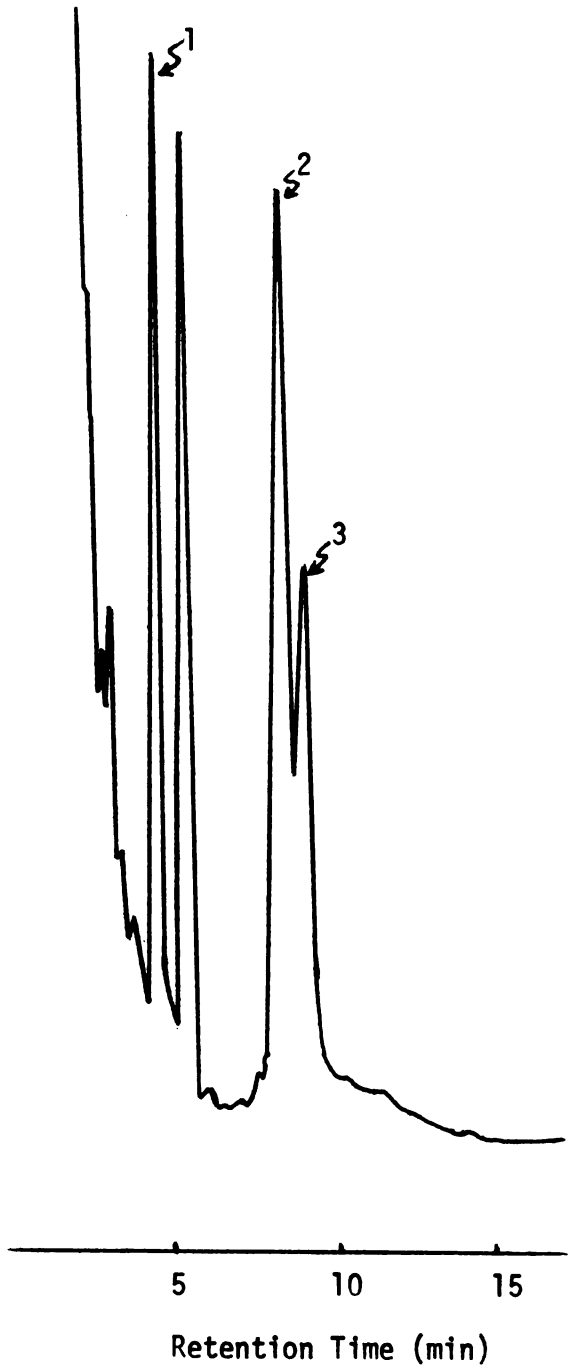


Fig. 3. Gas Chromatography: 3% OV-1 on Gas Chrom Q at 219° after Acetylation.

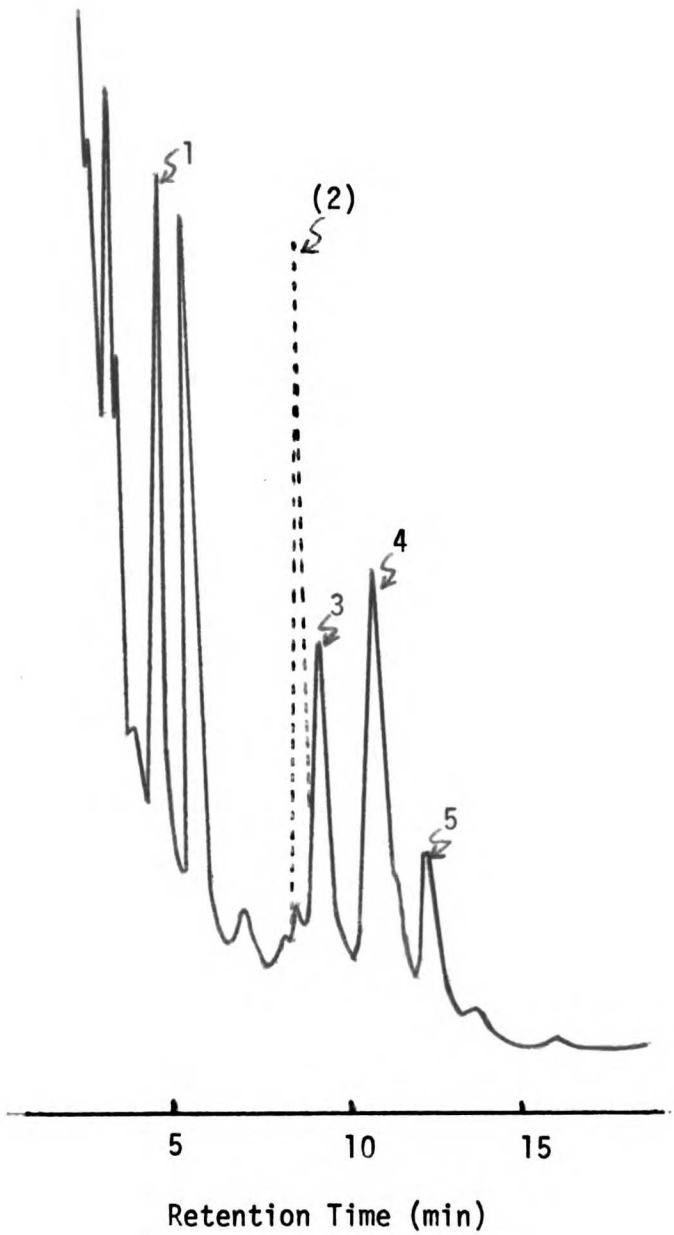
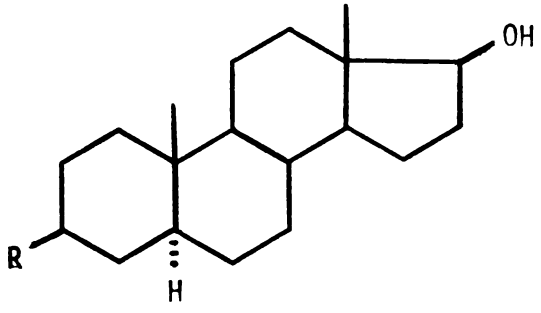


Table 2. Mass Spectrometric Data for 3-Oxygenated Metabolites.



Peaks (m/e)			
3α-AcO and 3β-AcO		3-keto	
348	Parent Peak	304	Parent Peak
333	M - CH_3	289	M - CH_3
330	M - H_2O	286	M - H_2O
315	M - (CH_3 + H_2O)	271	M - (CH_3 + H_2O)
288	M - HOAc	244	
270	M - (HOAc + H_2O)	229	
255	M - (HOAc + H_2O + CH_3)	218	

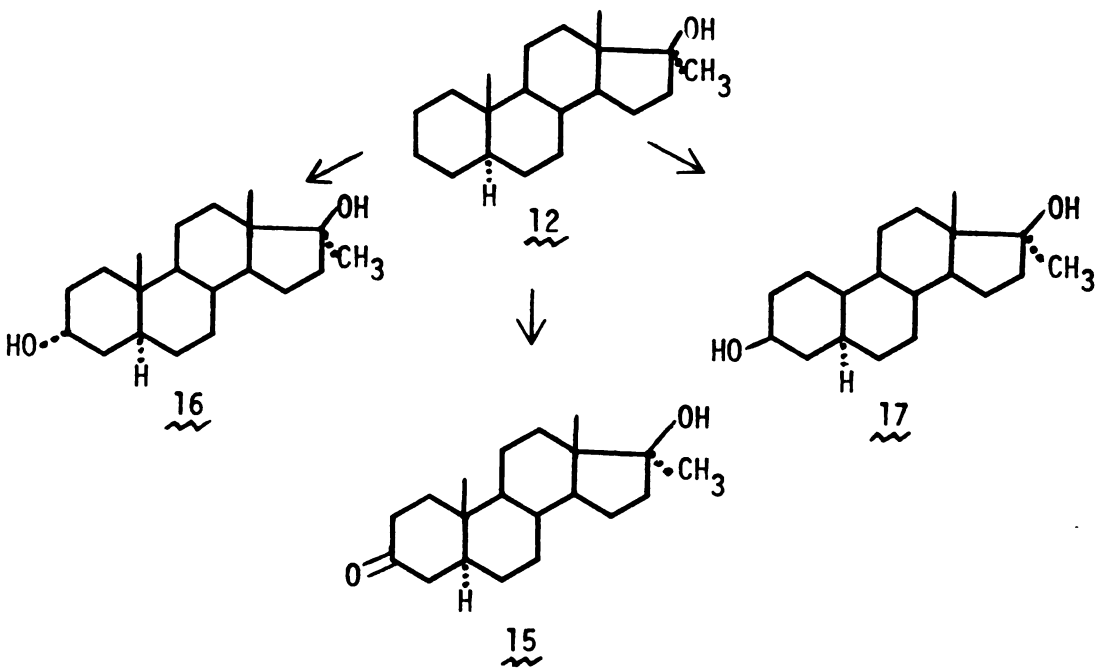
these metabolites with the use of the authentic compounds, thus identifying the compounds.

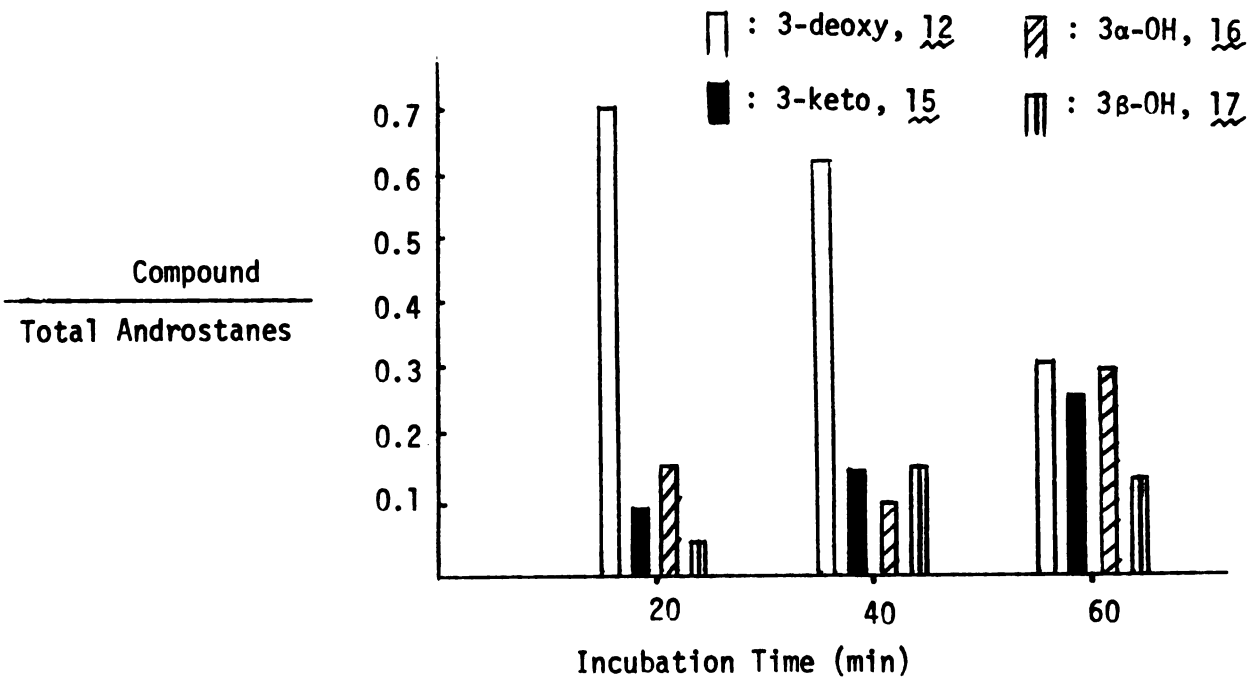
Quantitative analysis of the unchanged parent compound and its C-3 oxygenated metabolites was carried out by measuring the areas under the respective gas chromatographic peaks. From Fig 4, it is seen that the extensive 3-oxygenation of 12 occurs in rabbit liver homogenate. The ratio of the total amount of corresponding 3-oxygenated products 15, 16, and 17 in the incubation mixture to unchanged parent compound 12 is 2 : 1 after 60 minutes incubation under the conditions of the experiment.

In the latter part of the experiment, mutual interconversion of 3-oxygenated products, i.e., 3-keto 15 (17α-methyl-5α-androstan-3-on-17β-ol), 3α-OH 16 (17α-methyl-5α-androstane-3α,17β-diol), and 3β-OH 17 (17α-methyl-5α-androstane-3β,17β-diol) derivatives was investigated.

Incubation of the 3-keto derivative 15 shows a rapid conversion of this steroid to the corresponding 3-OH products 16 and 17. That is, the ratio 3-keto/total androgens in the incubation mixture is 0.3 after 15 minutes (Fig 5). Fig 6 shows that the 3α-OH derivative 16 disappears very quickly from the incubation mixtures during the first 60 minute period. The curve of rapid disappearance of this steroid, however, is markedly retarded and levels off after this period. Although the conversion of 3-keto 15 and 3α-OH 16 to the corresponding metabolites in question takes place to a large extent during the incubation period studied, it is clearly seen from Fig 7 that the metabolic conversion by rabbit

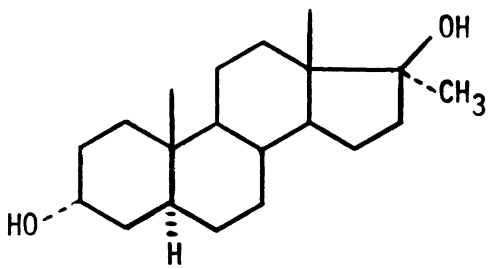
Fig. 4. Formation of 3-Oxygenated-17α-methyl-5α-androstan-17β-ols by Rabbit Liver Homogenate.



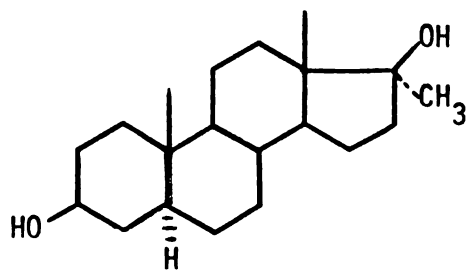


Incubation of rabbit liver homogenate with 12.

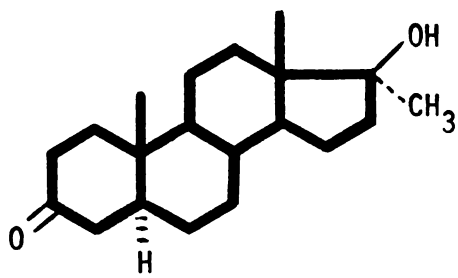
Fig. 5. Interconversion of 3-Oxygenated-17α-methyl-5α-androstan-17β-ols on Incubation with 15.



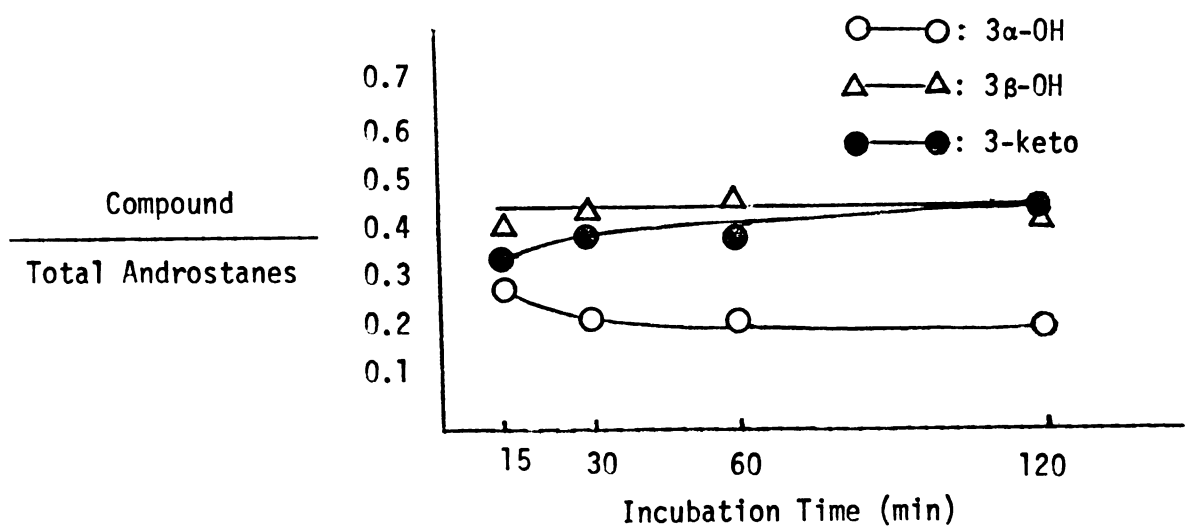
16



17

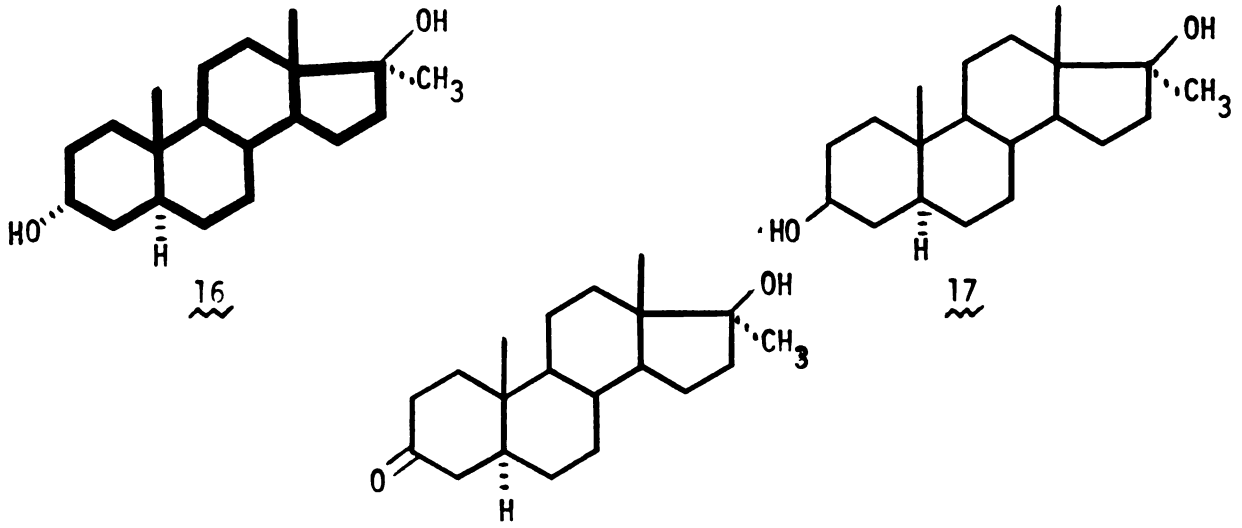


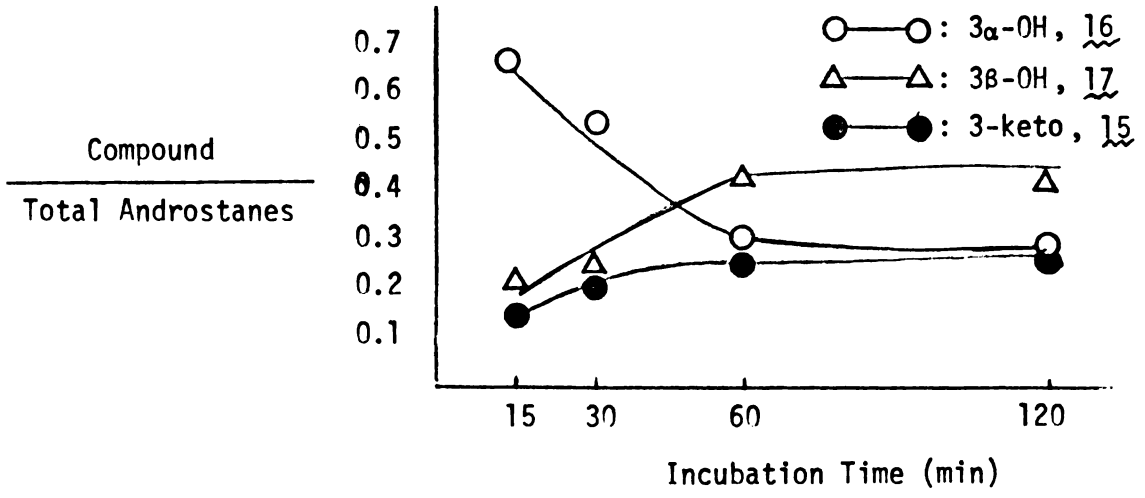
15



Incubation of Rabbit Liver Homogenate with 15.

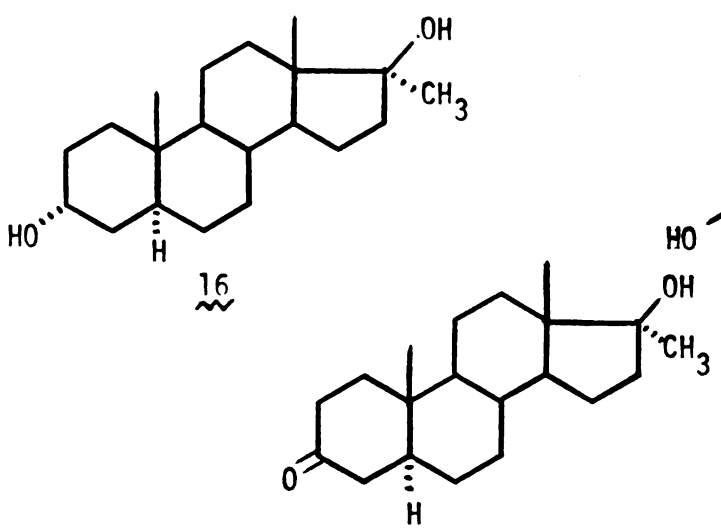
Fig. 6. Interconversion of 3-Oxygenated-17α-methyl-5α-androstan-17β-ols on Incubation with 16.

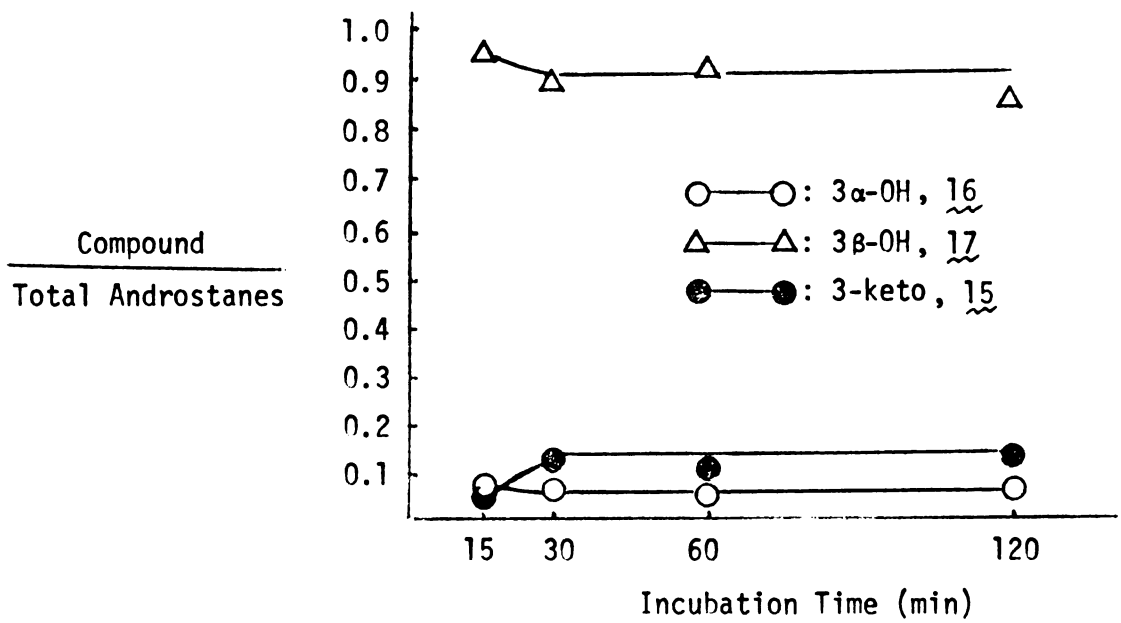




Incubation of Rabbit Liver Homogenate with 16.

Fig. 7. Interconversion of 3-Oxygenated-17α-methyl-5α-androstan-17β-ols on Incubation with 17.





Incubation of Rabbit Liver Homogenate with 17.

liver homogenate of 3β-OH 17 to the corresponding 3α-OH 16 and 3-keto 15 products proceeds only to a minor extent. In other words, the 3β-OH androstane derivative 17 is not converted efficiently to potent androgens 3-keto 15 or 3α-OH 16.

Discussion

The introduction of oxygen function into A ring of 17α-methyl-5α-androstan-17β-ol 12, with consequent formation of a carbonyl or hydroxyl substituent in the C-3 position, greatly affects biological activity of the resulting molecules. Kochakian (8) studied the effects of the oral administration of 12 and observed that this compound was highly active in promoting growth of the castrated mice prostate and seminal vesicles. In his assay, it was also found that the relationship of the chemical group at the position 3 to androgenic activity after oral administration of 17α-methyl androgens to mice was given the following sequence:

C=O > 3α-OH > 3β-OH > no group.

Nutting et al. (9) reported that 17β-hydroxy-17α-methyl-5α-androstan-3-one 15 and 17α-methyl-5α-androstane-3α,17β-diol 16 showed 50 to 60% and 65 to 70% activity of 17α-methyltestosterone, respectively, after oral administration of these compounds to castrated rats, while the effect of intramuscular injection of 17α-methyl-5α-androstane-3β,17β-diol 17 to castrated rats was not significant in comparison to 15. Dorfman and Kincl (11) found that on subcutaneous injection to rats 15 was 2.5 times more active than testosterone in its ability to increase the weights of the seminal vesicles and of ventral prostate. They also found that 17α-methyl testosterone administered by gavage was 2 to 3 times as active as testosterone, whereas the androgenic activity of these two compound was at the same level when administered by subcutaneous injection to rats.

Since reduction-oxidation processes are very important ia sll living tissues, all ketone and secondary hydroxyl groups are susceptible to redox conversions. This factor is extremely important with respect to the 17-keto-17-hydroxyl group transformation. As has already been discussed in the previous section B-iii, the reversible abstraction of hydrogen at C-17 from testosterone has been demonstrated. Huggins and Jensen (2) pointed out earlier that the state of oxidation of the chemical group at C-17 is critical in determining biological activity and that whereas the presence of 17β-hydroxyl group conferred considerable activity, compounds with a ketone at C-17 were devoid of this capacity. Testosterone, which possesses a 17β-hydroxyl group, is known as a potent natural androgen and androstenedione (5α-androst-4-ene-3,17-dione), which possesses a 17-ketone group, is a very weak one; therefore the protection of the 17β-hydroxyl group against oxidative metabolic inactivation becomes a very important consideration. Since compounds administered orally must pass through the liver, the fraction of the dose reaching to the general circulation may be considerably reduced compared to parenteral administration. Thus the differential oral potency of 17α-methyltestosterone and testosterone can be explained on the basis the 17α-methyl group of 17α-methyltestosterone preventing its 17β-hydroxy from forming the inactive 17-keto derivative. Introduction of a 17α-alkyl substituent into the testosterone molecule changes the secondary 17β-hydroxyl group to a tertiary hydroxyl group. Thus metabolic inactivation through oxidation of the 17β-hydroxyl group

to a 17-keto group is no longer possible and there would not appear to be any metabolic process whereby the 17-alkyl group can be removed. Therefore, it is reasonable to assume that the 17α-alkyl testosterone derivatives, or in general 17α-alkyl substituted androgens, owe their oral activity to the protection of the 17β-hydroxyl group.

In connection with the importance of oxygen function at C-3 of androgens, it is of interest to note that 5α-androstan-17β-ol 11 was found to be twice as androgenic as testosterone on inunction of the chick's comb (44) and equally as active on oral administration to the immature rat (6). The relatively high androgenic activity of 11, using a highly purified compound, was also demonstrated by Dorfman et al. (7) in a chick comb test by inunction. Cavallero and Ofner (45) studied the relative effectiveness of various steroids in an androgen assay using the exorbital lacrimal gland of the castrated rat and found that 11 was only 4.8% as active as testosterone. Their bioassay results for the isomeric diols demonstrated that the equatorial 3β-hydroxy steroid, 5α-androstane-3β,17β-diol, showed very low activity, whereas the axial 3α-hydroxy compound, 5α-androstane-3α,17β-diol was 3 times as potent as testosterone. Similarly, on direct application to chick comb, the 3α-OH isomer was also shown to produce high androgenic activity (44) and the effect of the 3β-OH isomer was relatively small on the rat seminal vesicles and ventral prostate indices after intramuscular or oral administration (46). The 3-keto derivative, 17β-hydroxy-5α-androstan-3-one, was found to be slightly less active than

testosterone under the conditions of the exorbital lacrimal gland assay (45). 17β-hydroxy-5α-androstan-3-one, on the other hand, was shown to be as active (46) or 1.5 to 3 times as active as testosterone (11,13,47) on the basis of the weight increase of rat ventral prostate and seminal vesicles. Recently Goldman and Baker (48) determined the degree to which various androgens increase the anogenital distance in the female rat fetus and found that the virilizing potency in order of decreasing effectiveness was 17β-hydroxy-5α-androstan-3-one > 5α-androstane-3α,17β-diol > testosterone > 5α-androstane-3β,17β-diol.

In relation to the difference in androgenic activity of 3-oxygenated androgens, it is known from both chemical (49) and microbiological work (50) that the 3α-alcohols (axial epimers) are oxidized more readily to the corresponding 3-ketone than are the 3β-alcohols (equatorial epimers), in accordance with the thermodynamic prediction (see, for example, 51). The possibility, therefore, exists that *in vivo* the 3-hydroxy-5α-androstanes are oxidized in various degrees to the even more potent androgens, the 3-keto-5α-androstanes, and that, in fact, the 3-alcohols may be androgenically inactive *per se*. In this connection, an interesting observation reported by Ringold et al. (52) is that 17α-methyl-5α-androstane-3β,17β-diol 17 was 3.4 times more active than its 3α-deutero compound on the basis of the ventral prostate and 4.7 times more active on the basis of the response of the seminal vesicles, demonstrating that in the rat transformation to the 3-keto form is an important factor contributing to

androgenic activity of 3β-hydroxy-5α-androstanes. In other words, if the activity of the 3β-OH compound depends on oxidation to the 3-keto with loss of the 3α-hydrogen as the rate limiting step, then, in accord with the primary deuterium isotope effect, the 3α-deutero compound should be oxidized more slowly and consequently would be expected to exhibit lower androgenic activity than the 3α-hydrogen compound.

It is also known that in the castrated rat assay 3α-methyl-5α-androstane-3β,17β-diol and 3β-methyl-5α-androstane-3α,17β-diol were found to be completely devoid of androgenic activity (53). As in the case of a 17-methyl-17-OH compound, reversible biological oxidation of the 3-OH group is impossible and could account for the inactivity of these compounds.

In the present study, the ability of rabbit liver to oxygenate the position 3 of 17α-methyl-5α-androstan-17β-ol 12 has been demonstrated. As is clearly seen in Fig 4, extensive 3-oxygenation of 12 occurs in rabbit liver homogenate and the ratio of the total amount of the corresponding 3-oxygenated products in the incubation mixture to the unchanged parent compound is 2 : 1 after 60 minute incubation.

As has been discussed above, references to the androgenicity of 3-oxygenated androstanes indicate that 3-keto- and 3α-hydroxy-5α-androstan-17β-ols show more potent androgenic activity than testosterone, while 3β-hydroxy derivative is not a potent androgen. The fact that the 3-oxygenated products, especially 3-keto and 3α-hydroxy derivatives, of 12 were detected in significant quantities accounts for some or possibly

all of the androgenicity of ring A hydrocarbon androstanes, such as 17α-methyl-5α-androstan-17β-ol or 5α-androstan-17β-ol, in various target tissues.

It is obvious from the data presented in Figs 5, 6 and 7 that the time sequence of the mutual interconversion of 17β-hydroxy-17α-methyl-5α-androstan-3-one 15, 17α-methyl-5α-androstane-3 ,17β-diol 16, and 17α-methyl-5α-androstane-3β,17β-diol 17 shows quite different patterns. Although the conversious of 15 or 16 to the other two metabolites in question takes place to a large extent during the incubation period studied as shown in Figs 5 and 6, it is clearly seen from Fig 7 that the metabolite conversion by rabbit liver homogenate of 17 to 15 and 16 proceeds only to a minor extent. That is, the 3β-hydroxy androstane derivative 17 is not converted effectively to potent androgens 17β-hydroxy-17α-methyl-5α-androstan-3-one 15 and 17α-methyl-5α-androstane-3α, 17β-diol 16.

In view of the high androgenic potency possessed by 3α-hydroxy- and 3-keto-5α-androstan-17β-ols, the results obtained from the present study explain the androgenic activity of 3-deoxy-5α-androstan-17β-ol in terms of metabolism to active metabolites. Furthermore, the differences in the metabolic fate of 3-oxygenated derivatives 3-keto 15, 3α-hydroxy 16, and 3β-hydroxy 17 are in keeping with the differences in androgenic activities of these steroids.

Robel et al. (54), however, recently reported that 3β-androstanediol

was an active androgen when tested in organ cultures and its action was attributed to its own intrinsic activity. It should also be pointed out here that studies by Sydnor (3) on the ability of 3β-fluorosteroids, 3β-fluoro-5α-androstan-17β-ol, to promote growth of accessory sexual tissues in the hypophysectomized female rat have shown that fluorine can replace a hydrogen atom or hydroxyl function at C-3 without appreciable loss of biological activity.

Bloom and Shull (55) demonstrated the enzymatic epoxidation of non-conjugated unsaturated steroids by microorganisms in addition to previously recognized hydroxylation at saturated centers. According to their observation for oxidative attack on steroids, including hydroxylations and epoxidations, a microorganism capable of introducing an axial hydroxyl function at C_n of a saturated steroid also effected the introduction of an epoxide grouping at C_n in the corresponding unsaturated substrate. Equatorial hydroxylases did not effect a similar conversion. Bloom (56) assumed that hydroxylations proceed by electrophilic attack.

In connection with Bloom's proposed mechanism for oxidative attacks on steroids, it was thought to be of interest to investigate the possibility of C-3 oxygenation of 17α-methyl-5α-androst-2-en-17β-ol 14 by the same *in vitro* system used for 17α-methyl-5α-androstan-17β-ol 12. 17α-methyl-5α-androst-2-en-17β-ol 14 is known to possess relatively high androgenic activity. On the basis of increase in weights of rat ventral prostate and seminal vesicles, this compound was shown to produce andro-

genic activity of 120 to 200% of 17α-methyltestosterone when administered orally (9) and 60 to 90% of testosterone by subcutaneous injection (11). In the chick's comb assay by local inunction (4), however, 14 exhibited 20% activity of testosterone.

By contrast to the expectation that 14 would also give extensive oxygenation at C-3 under the same experimental conditions employed for 12, there was found to be no significant metabolic conversion of this androgen.

Since the in vitro metabolic studies of 17α-methyl-5α-androstan-17β-ol 12 alone do not provide a definite guide to the active form of the androgen, studies of the in vivo uptake of the C-3 oxygenated products of 12 by rabbit ventral prostate was next undertaken. Such knowledge is of potential use in the interpretation of metabolism data and other indications of the significance of metabolic transformation would come from such uptake studies. These are discussed in the following section.

PART II. *IN VIVO* DISTRIBUTION AND *IN VITRO* UPTAKE AND RETENTION OF ANDROGEN ANALOGS IN RABBIT VENTRAL PROSTATE

A. INTRODUCTION

The availability of high specific activity labeled steroids has prompted many studies on the uptake and distribution of these hormones in various tissues. Target organs for various sex hormones are generally able to take up the circulating hormone preferentially. Male accessory sex organs, such as prostate gland and seminal vesicle, possess selective affinity for radioactive androgens. Among these organs, ventral prostate exhibit the highest uptake and retention of androgens (58-65).

Cyproterone acetate, a potent anti-androgen, causes significant decreases in the uptake of radioactivity by ventral prostate (66-71) and by various other tissues (72) following the injection of ^{3}H-testosterone to male rats pretreated with the anti-androgen. Several other hormonal compounds, such as androstenedione, 17α-methyl-testosterone, 17α-methyl-nor-testosterone, progesterone and corticosterone, influence the ^{3}H-testosterone uptake by rat ventral prostate (73).

The investigations described above on the localization and uptake of androgens in various organs are important in demonstrating selective target organ retention of these hormones. The metabolism of androgens, in particular testosterone, by the target organ also has been the subject of extensive studies. These studies are important in finding the "active forms" of the hormones and in finding whether there was some cause and

effect relationship between hormone metabolism in target tissues and hormone action (59, 74-87). Several types of evidence from these studies suggest that the 5α-reduced derivative of testosterone, 17β-hydroxy-5α-androstan-3-one (dihydrotestosterone), is the active form of the hormone at the nuclear sites of action within certain accessory organs of male reproduction.

The next approaches to finding the "receptor" involve the studies of the interaction of testosterone or its metabolites with macromolecules in target organs. Bruchovsky and Wilson (80), and Anderson and Liao (81) in 1968, and Mainwaring (88) in 1969 studied the nuclear androgen-receptor in prostatic tissue. During 1969, the existence of a specific 5α-dihydrotestosterone-binding protein in the 105,000 g supernatant fraction of homogenates of rat prostate was reported independently by several research groups (89,90). Since then, a number of investigators have studied the formation of androgen-macromolecular complexes in both nuclei and cytoplasm of the target organ and special attention has been given to specific proteins involved in this process (89-97).

In the present study, an attempt was made to examine further the possibility that a 5α-androstane derivative having a simple hydrocarbon A-ring, 17α-methyl-5α-androstan-17β-ol 12 owes its androgenicity to metabolic oxygenation at C-3 and to see whether or not 17α-methyl-5α-androst-2-en-17β-ol 14 itself is responsible for eliciting the androgenic response. In this approach, the *in vivo* distribution of the steroid and its metabolites in the ventral prostate of male rabbits was examined

following the administration of each of these two synthetic ^{14}C-labeled androgens. Particular attention was given to the occurrence of the C-3 oxygenated products of these androgens in the target organ in question.

Studies of the uptake and retention of radioactively labeled androgens, 17α-^{14}C-methyl-5α-androstan-17β-ol ^{14}C-12, 17α-^{14}C-methyl-5α-androst-2-en-17β-ol ^{14}C-14, and 1,2-^{3}H-17β-hydroxy-5α-androstan-3-one ^{3}H-2, by rabbit ventral prostate slices were next carried out to determine whether or not the binding sites for these hormones are identical. The influence of various non-labeled steroids on the retention of labeled androgens by ventral prostate slices was also studied. The aim was to describe these events in terms of the nature of the active sites of primary receptors for these androgens in relation to the structure-activity relationships for androgenic hormones.

B. GENERAL CONSIDERATIONS: Metabolism and Protein Binding of Androgens in Target Organ

i. Uptake and selective retention of androgens by target organs.

The dramatic changes in the size and functions of responsive tissues which result from androgen treatment of juvenile, orchiectomized or hypophysectomized mammals in postnatal life has stimulated much work over the last two decades on metabolic and enzymic events in these organs. The metabolic parameters of androgen action have been reviewed by Williams-Ashman (98) and these include cell respiration, synthesis of nucleic acids and of proteins, and incorporation of amino acids into proteins.

A study of the ability of target organs to specifically bind 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 of radioactively labeled androgens by the target organs, particularly by the rat ventral prostate. Greer (57) and later Lawrence (58) found a significantly higher accumulation and retention of radioactivity in ventral prostate and seminal vesicles compared to muscular tissue after injecting ^{14}C-testosterone to rats. A selective retention of androgen by these target organs is indicated, since the pattern of uptake did not follow that of blood. Harding and Samuels (59) and Tveter and Attramadal (60) also demonstrated in rats that tissue radioactivity after the injection of radioactive testosterone is higher in the ventral prostate than in plasma and other non-target tissues such as liver and muscle. In addition,

Harding and Samuels (59) found that blood or liver contains large amounts 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 of conjugated androgens. Roy and Laumas (61) carried out a comparative study of the uptake of radioactivity in the genital, neural and other tissues of intact male, castrated male and female rats after constant infusion of ^{3}H-testosterone. It was found that castration had a marked effect in enhancing the uptake of radioactivity in the different tissues, particularly liver and kidney which have a metabolic and excretory role. Prostate and seminal vesicles, on the other hand, did not show any difference in uptake between intact and castrated rats. In experiments on the subcellular localization of radioactivity it was found that prostate and seminal vesicles had heavy localization in the nuclear and 105,000 g soluble fraction, whereas the major localization of radioactivity in the case of non-target tissue like liver, intestine, muscle, etc., was in the soluble fraction.

Tveter (62) studied the distribution of radioactivity in the different lobes of the rat prostate following the intramuscular injection of ^{3}H-testosterone. A selective uptake of radioactivity relative to muscle was found in all parts of the prostate, i.e., in ventral, dorsal and lateral prostatic lobes and in the coagulating gland. Eight hours after the injection, the radioactivity in the lateral prostate was about 24 times higher than in muscle, the corresponding ratios for the ventral and dorsal lobese being 20 : 1 and 11 : 1, respectively. After the intramuscular

injection of ^{3}H-testosterone to adult castrated male rats (62), a high and prolonged accumulation of radioactivity was found in the prostate in contrast to the much lower uptake by muscle. In the ventral prostate, the uptake was 205% higher in animals castrated 24 hours previously than in non-castrated animals. The corresponding values for the lateral prostate, the coagulating glands and seminal vesicles were 120%, 165%, and 213%,respectively. The uptake by muscle was not influenced by castration. Following homogenization of the coagulating gland and the dorsal and ventral prostate, it was demonstrated that some of the radioactivity in the 105,000g supernatant fraction was associated with macromolecules. Thomas et al. (64) reported that substantial amounts of radioactivity accumulated in the anterior lobes of the prostate gland after single injections of ^{3}H-testosterone to normal mice. Levels of radioactivity were considerably higher in organs obtained from mice injected by the intraperitoneal route than by subcutaneous injections.

Hansson (65) studied the uptake and retention of testosterone and 5α-dihydrotestosterone by rat ventral prostate in vitro. It was found that the radioactivity in the prostate was about four times higher than in the muscle after the incubation of slices of ventral prostate and skeletal muscle from castrated rats with ^{3}H-testosterone. The addition of non-labeled testosterone to the incubation medium reduced the prostatic retention of androgen almost to that in the muscle specimens. Incubation with ^{3}H-dihydrotestosterone gave essentially the same results as incubation with ^{3}H-testosterone.

Neuman and von Berswordt-Wallrabe (66) suggested that action of a potent anti-androgen, cyproterone acetate, is one of competitive inhibition with testosterone or with its metabolites at the level of the target organ. It was also reported that cyproterone acetate and cyproterone strongly inhibited the uptake of radioactive androgens by ventral prostate and seminal vesicles (67-71). In the case of ventral prostate, it was shown that cyproterone acetate inhibits the retention of 5α-dihydrotestosterone by prostatic nuclei *in vivo*(67,68,70,71). This inhibitory effect of the anti-androgen on the retention of dihydrotestosterone has also been shown in *in vitro* tissue immersion experiments (70,71,72). Since it was demonstrated that such inhibition can be observed when minced prostate is incubated with ^{3}H-dihydrotestosterone (73) and that cyproterone has only a slight effect of testosterone metabolism (68), the inhibition is not the site of reduction of testosterone to dihydrotestosterone.

Tveter and Aakvaag (73) made a study of the influence of certain hormones and synthetic hormonal compounds on the uptake of radioactivity by rat seminal vesicles and prostate gland following the administration of ^{3}H-testosterone *in vivo*. It was shown that androstenedione, 17α-methyl testosterone, 17α-methyl-β-nor-testosterone (SK & F 7690), progesterone, and corticosterone reduced the uptake of radioactivity. However, 17β-estradiol and diethylstilbestrol did not reduce the uptake; indeed, in the lateral prostate an increased accumulation of radioactivity was observed.

Fang, Anderson and Liao (70) demonstrated that cyproterone acetate suppressed the uptake of radioactive androgens by rat ventral prostate

in vivo and this was accompanied by a decrease in the retention of 5α-dihydrotestosterone by prostatic cell nuclei. It was shown that cyproterone and its 17α-acetate inhibited the formation of a specific dihydrotestosterone-protein complex in prostatic cell nuclei when minced prostate was incubated with radioactive testosterone or dihydrotestosterone. 17β-Estradiol, diethylstilbestrol, and progesterone, but not cortisol, also suppressed the retention of dihydrotestosterone by prostatic cell nuclei in vitro, but to a much lesser extent than cyproterone acetate. This suppressive effect of these compounds appears involved in the antagonistic action of anti-androgens on the androgen dependent growth of accessory reproductive organs. Belham and Neal (71) also reported that diethylstilbestrol, a non-steroidal anti-androgen, inhibited the nuclear retention of dihydrotestosterone when both ^{3}H-testosterone and diethylstilbestrol were injected intraperitoneally in vivo, but no effect on dihydrotestosterone retention when both testosterone and diethylstilbestrol were supplied directly to the prostate either in vivo or in vitro. Therefore, if diethylstilbestrol has anti-androgenic effect at the level of target organ as distinct from its effect on androgen production by the testis, it is probably due to a mechanism differing from that of cyproterone and its acetate.

Although further investigations are needed to look into the mode of action of anti-androgens, it is apparent that testosterone or its metabolites is accumulated and selectively retained in the target organ.

ii. Testosterone metabolism in the rat ventral prostate.

The natural androgens and some of synthetic androgens undergo a series of metabolic transformation not only in organs such as the liver, but also in peripheral tissues such as the male accessory genital glands which are classical target organs for androgenic hormones.. What is the relation of such metabolic alterations of androgens to their mode of action and the nature of the "active forms" of testosterone and other androgens in target tissue? In target cells, if there is no hormone metabolism, it follows that the steroid is active per se, as in the case for estradiol in the atrophic uterus (74). The growth of the accessory sex organs of the immature male mammals is controlled in large part by testosterone. Although this biological event has been attributed to the effects of testosterone itself, such an interpretation is obscured by the fact that testosterone is extensively metabolized in vivo. Many interesting studies of testosterone metabolism in the prostate have been conducted during the last two decades. Experiments in vivo by a single injection of testosterone allowed for the identification of metabolites in the prostate (59,75), but not for the definite proof of their formation in situ. In vitro short incubation experiments indicated the presence of certain metabolic enzymes in the prostate (76,77,78). Some of the testosterone metabolites found in the prostate in the in vivo and in vitro experiments exhibit noticeable androgenic properties (99). With reference to the prostate weight, certain 5α-androstane derivatives have been found to be active androgens, 5α-dihydrotestosterone and 5α-

androstane-3α,17β-diol at least as active as testosterone or more (99). the action of testosterone could, therefore, be caused directly by the hormone, or indirectly through its transformation into metabolites.

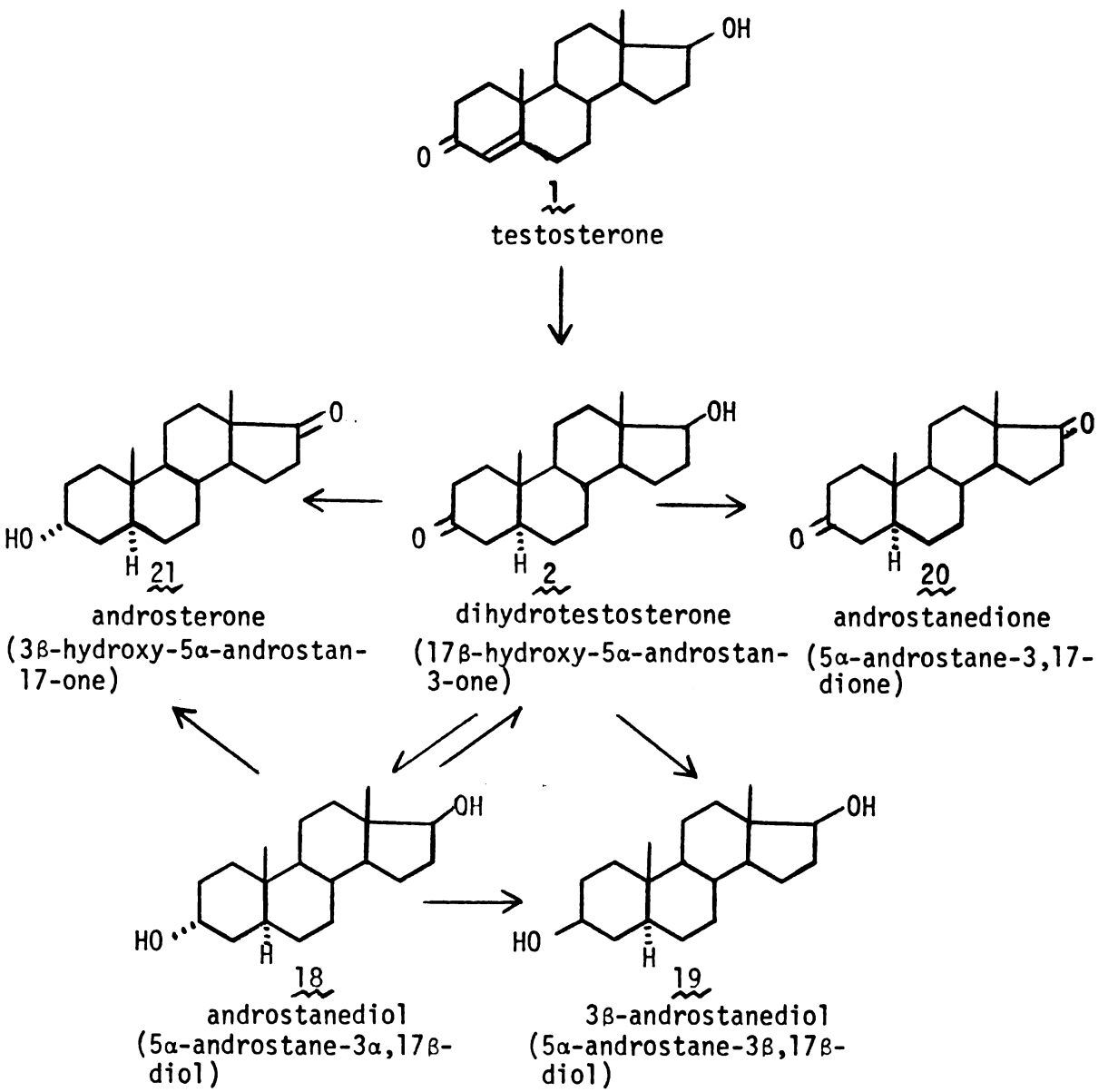
In 1968, two research groups suggested that dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) might be an active form of testosterone in some androgen-sensitive organs and appears to associate selectively with nuclear components of these tissues. Bruchovsky and Wilson (79,80) found that within one minute following the intraveneous administration of ^{3}H-testosterone into normal and hepatectomized rats, testosterone was taken up by the prostate and at least 90% was converted to three products, 5α-dihydrotestosterone 2, androstanediol 18, and androsterone 21. Those metabolites were demonstrable in the cytoplasm of the ventral prostate within a few minutes, whereas only testosterone and dihydrotestosterone were recovered from prostatic cell nuclei for periods as long as two hours after testosterone injection. Furthermore, Bruchovsky and Wilson (79) showed that in the presence of a NADPH-generating system, prostatic nuclei converted testosterone to dihydrotestosterone, whereas prostatic cytoplasm, in addition, reduced dihydrotestosterone to androstanediol 18. It was also shown that as early as 15 minutes after testosterone administration the radioactivity recovered in the bound form was predominantly dihydrotestosterone and that this metabolite was bound to an acidic nuclear protein, thought to be of chromatin origin according to the experimental data (80). Thus, testosterone is reduced in the nuclei of androgen-sensitive tissues to 5α-dihydrotestosterone, which appears to function as

an "active form" of the hormone.

Anderson and Liao (81) demonstrated independently that in the prostatic cell nuclei of rats injected with ^{3}H-testosterone, 5α-dihydrotestosterone represented the major (>75%) radioactive product and testosterone was a monor (5-25%) component: No other radioactive metabolites were detected in the nuclei. On the other hand, in the cytoplasmic soluble fraction less than 5% of total radioactivity was in the form of free dihydrotestosterone. A number of radioactive metabolites, such as androstanedione 20 and other compounds more polar than testosterone were found in the cytoplasm. When liver nuclei and the cytoplasmic fraction of the same rats were analyzed for comparison, there was found to be no selective retention of dihydrotestosterone by liver nuclei, although many metabolites of testosterone were demonstrable in the liver cytoplasm. Anderson and Liao (81) also showed that the selective binding of dihydrotestosterone by rat prostatic nuclei in vivo can be reproduced in vitro by incubating minced prostate glands with ^{3}H-testosterone. When cell nuclei were isolated from prostatic cells at the end of the incubations, only dihydrotestosterone (75-90%) and a small amount of testosterone could be identified. Under these conditions, at least five other metabolites were demonstrable in the prostatic cytoplasm. Such selective retention of dihydrotestosterone by nuclei of minced tissues in vitro was not observed with thymus, brain, diaphragm, and liver. These experiments raise the possibility that testosterone is active through the formation of potent metabolites in the target organ.

To test this possibility, Baulieu's group (82,83,84) investigated the metabolism of testosterone and its metabolites by rat ventral prostate gland in organ culture, in order to examine testosterone metabolism and the action of the hormone and its metabolites in an isolated system. Baulieu (82) pointed out that there was no suitable *in vivo* system for testing the hypothesis with regard to the testosterone action, since the same testosterone metabolites found in the prostate are formed by the liver and in other organs and may reach the target organ via the blood, making the system too complicated for physiological analysis. Furthermore, he pointed out that *in vitro* incubation experiments of slices or homogenates were not appropriate either to study the problem, since no response to hormone has been observed under such conditions, and steroid metabolism may differ greatly from that found *in vivo*. In the experiments carried out by Baulieu's group (82,83,84), it was shown that in the rat ventral prostate tissue culture, there was metabolism of testosterone to several 5α-reduced compounds, 5α-dihydrotestosterone 2, the 3α,17β- and 3β,17β-diols 18 and 19, and androsterone 21 (see, Fig 8). The results are essentially the same as those obtained from *in vivo* and *in vitro* experiments carried out by Wilson (79,80), Liao (81) and Belham et al. (85). In the organ culture experiments, dihydrotestosterone, the main metabolite, formed the greatest proportion of total radioactivity in the nuclei and was also the most abundant identified metabolite in the cytoplasmic fraction and in the medium. Tveter and Aakvaag (86) also showed that in the *in vivo* experiments

Fig 8. Metabolism of Testosterone in the Rat Ventral Prostate (79-86).



5α-dihydrotestosterone was the main metabolite after intramuscular injection of ^{3}H-testosterone to adult castrated male rats, representing 49 - 72% of the total radioactivity in the ventral, dorsal and lateral prostate, the coagulating glands and the seminal vesicles, whereas the values for unchanged ^{3}H-testosterone were 6 - 16%.

The 5α-reductase necessary for the conversion of testosterone to 5α-dihydrotestosterone was reported to be associated with the nuclei (79,80, 81) and the cytoplasmic particles (78). However, Baulieu (83) found that a greater activity for 5α-reductase was in the microsomes than in the nuclei obtained from the same amount of tissues. The presence of a 3α-keto-steroid oxidoreductase in the rat ventral prostate cytoplasm was demonstrated by Unhjem (100).

Baulieu's group (83,84) also studied the metabolisms of metabolites prepared in radioactive form. 5α-Dihydrotestosterone was not converted to testosterone, but otherwise the pattern of metabolites in the cytoplasm and nuclei was similar to that of testosterone. Androstanediol 18 was largely converted to 5α-dihydrotestosterone and androsterone 21 and to a lesser extent to 3β-androstanediol 19. In contrast, 3β-androstanediol 19 was not converted to 5α-dihydrotestosterone, androsterone 21 or androstanediol 18; its only metabolite identified in the cytoplasm and nuclei was epiandrosterone (3β-hydroxy-5α-androstan-17-one). Moreover, the activity of testosterone and its metabolites was tested in organ culture (83,84). These results provided evidence for some correlation between steroid metabolism and action in the rat ventral prostate in

culture. Testosterone, 5α-dihydrotestosterone, and the 3α,17β-diol exhibited essentially the same type of activity, hyperplasia and secretion. As discussed above, those three compounds are transformed into the same metabolites with the exception of the lack of transformation of 5α-dihydrotestosterone and the 3α,17β-diol into testosterone, which indicates further that testosterone is not necessarily a "hormone". Conversely, the 3β,17β-diol provoked hypertrophy and secretion but not hyperplasia, and was not metabolized into 5α-dihydrotestosterone.and the 3α,17β-diol. It is interesting to note that under these conditions, the 3β,17β-diol, which has been considered inactive (99), has been found to have a definite androgenic activity and that the different reactions provoked by 5α-dihydrotestosterone and the 3α,17β-diol are observed.

In order to extend these observations, Bruchovsky (87) compared the metabolites formed in rat prostate following the in vivo administration of seven natural androgens including testosterone, 5α-dihydrotestosterone, and androstanediol to establish that the formation of 5α-dihydrotestosterone is a general characteristic of androgen metabolism in prostate. Every androgen tested was shown to give rise to several metabolites in the prostatic cytoplasm and invariably one of these was 5α-dihydrotestosterone. Moreover, in the nuclei dihydrotestosterone was found to be the major metabolite. The fact that the administration of seven natural steroids is followed by the nuclear incorporation of dihydrotestosterone indicates that this phenomenon is also a general feature of the prostatic response

to androgens. Thus, it appears that virtually all the natural androgens possessing a significant degree of potency are metabolized to 5α-dihydrotestosterone in varying amounts.

These studies of testosterone metabolism strongly suggest that testosterone action on the ventral prostate is related to the formation of active metabolites. The discovery of prostatic binding proteins, which will be discussed in the following section, also supports the importance of metabolism in hormone action. However, there is still no conclusive evidence that testosterone is inactive by itself.

iii. Receptor proteins for androgens: Isolation of dihydrotestosterone binding proteins from prostatic nuclei and cytoplasm.

Hechter and Halkerston (101) have pointed 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 physicochemical details of interaction of the hormone with these receptor substances. It is very 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, relatively few types of such receptors are present on the surface of 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.

One of the approaches to examining the nature of sex hormone receptors has been to study the binding of radioactively labeled hormones of very

high specific activity to specific cellular constituents, either after injection of the labeled hormone or by incubation with isolated tissues. The subsellular distribution of hormones in target organ homogenates has led to the study of steroid binding, more especially in the cytosol and the nuclear fractions and this subject has been reviewed (16,102).

1. Nuclear binding proteins.

There is now an overwhelming body of evidence suggesting that all types of naturally occurring steroidal hormones are selectively accumulated and bound within the nucleus of responsive tissues on which they exert important biological effects (see, for example, 103,104,105). In keeping with this general concept, androgenic steroids are selectively accumulated in the nuclei of such androgen-dependent tissues such as the rat ventral prostate. A unique feature of the nuclear binding of androgens, however, is that a metabolite, 5α-dihydrotestosterone, rather than the parent hormone, testosterone, is bound within the chromatin from androgen-dependent tissue. A number of investigators have studied the tissue specificity of the nuclear binding, the characterization of the binding component and the intranuclear binding site.

Bruchovsky and Wilson (80) studied the intranuclear binding of radioactive hormone in prostate after intraveneous administration of ^{3}H-testosterone to rats. Nuclei obtained by sucrose density gradient were extracted with buffer containing 0.6 M NaCl, and the soluble radioactivity was

separated into bound and free fractions by gel filtration on Sephadex G-25 or G-200. By the 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-dihydrotestosterone was bound to an acidic nuclear protein. Moreover, seceral characteristics of this binding phenomenon were defined; the binding was stable to freezing for as long as 8 days, stable to short term incubation at 20^{o} but not at 37^{o}, and partially stable to repeated gel filtration on Sephadex.

Liao's group (70,81) demonstrated that the selective binding of 5α-dihydrotestosterone by prostatic nuclei *in vivo* could be reproduced *in vitro* by incubating minced prostate glands with ^{3}H-testosterone. Under these conditions, the following observations were made: There are about 2,000 molecules of dihydrotestosterone retained by each prostatic nuclei and the retention of the radioisotope at 0^{o} is insignificant, at least in part, because of the requirement of an enzymatic transformation of testosterone to 5α-dihydrotestosterone. At 37^{o} the maximal retention is obtained after incubation for 20 - 40 minutes. The presence of NaCN or dinitrophenol in the incubation medium reduces the extent of binding by about two-thirds. Addition of 10 to 100 fold non-radioactive testosterone, androstenedione, or dihydrotestosterone during homogenization and nuclear isolation does not influence the amount or type of steroid bound. The complex is rather stable at neutral pH, but at pH below 6 or above 10, about 50% of the radioactivity is released from nuclei, at 2^{o}. The stability of the complex is temperature dependent. At pH 5.8, no loss of the retained radioactivity

occurs until 30^{o} is reached, followed by a linear decline in radioactivity until the temperature reaches 45^{o}. At this temperature only 20% of the initial radioactivity remains bound to the nuclei.

Mainwaring (88) also studied the specificity of the binding of ^{3}H-testosterone to nuclei of various rat tissues *in vivo*. He found that a significant amount of radioactivity was retained in the nuclei of androgen-dependent tissues only, particularly the ventral prostate gland and that the bound radioactivity was only partially recovered as ^{3}H-testosterone; the remainder was identified as ^{3}H-5α-dihydrotestosterone. Furthermore efforts were made to characterize the binding component, or "receptor," in prostatic nuclei. On digestion of nuclei labeled *in vivo* with ^{3}H-testosterone, with enzymes of narrow substrate specificity, only trypsin released tritium suggesting that the receptor is a protein. On the basis of subfractionation studies of labeled nuclei, the receptor was shown to be an acidic protein. The androgen-receptor complex could be effectively extracted from the prostatic nuclei in 1 M NaCl. From the results of fractionation by gel filtration on agarose, the complex was shown to possess a molecular weight of 100,000 - 120,000. The specificity of the binding of steroids to such 1 M NaCl extracts *in vitro* was also investigated by the equilibrium dialysis procedure. Under these conditions, the specificity of the binding of ^{3}H-testosterone demonstrated *in vivo* could not be simulated. According to the author's opinion (88), chromatin is certainly involved in the formation of the steroid-receptor complex but its precise intranuclear localization cannot be determined by

biochemical procedures alone. Mainwaring (88) further pointed out that the specificity of the binding process in vivo cannot be satisfactorily explained by the presence of a nuclear binding protein alone which is specific for androgens. As will be seen, the overall specificty of the binding of androgens in the prostate is also dependent on protein(s) located, for example, in the cytoplasm.

Unhjem (91) also demonstrated that 5α-dihydrotestosterone was taken up and bound by the nuclei following incubation of rat ventral prostate homogenates with ^{3}H-dihydrotestosterone. Isolated macromolecules from sodium chloride extracts of the nuclei were believed to be at least partially responsible for the binding.

2. Cytoplasmic binding proteins.

Since extensively purified prostatic nuclei or chromatin alone are unable to retain dihydrotestosterone, it became clear that additional factors from outside the nucleus itself were implicated in the selective binding process. These have been subsequently identified as specific hormone binding proteins or "receptors" in the cytoplasmic or cell supernatant fraction of androgen-dependent tissues.

A number of groups of investigators established the presence of soluble estrogen-receptor proteins in the cytoplasmic or 105,000g supernatant fraction of estrogen-dependent tissues (see, for example, 106,107). If the mechanism of steroid hormone action proposed by Jensen et al. (107) is generally applicable, then a soluble androgen-receptor should be present in prostatic tissue in addition to the nuclear androgen-receptor. A search for

such proteins in the cytoplasmic fraction of rat ventral prostate has been made by many investigators.

Mainwaring (89) identified a thermolabile protein with the properties of a steroid "receptor" in the cytoplasmic or 105,000g supernatant fraction of the rat prostate. The author showed that the receptor had a particular specificity toward 5α-dihydrotestosterone and that testosterone was bound to a lesser extent but other steroids, including certain androgenic hormones such as androstenedione (androst-4-ene-3,17-dione) and epiandrosterone (3β-hydroxy-5α-androstan-17-one), were not bound. The soluble androgen-receptor has a molecular weight of 274,000 and a sedimentation coefficient of 8.0 S, clearly distinguishing the receptor from the androgen-binding globulin in serum and the androgen-receptor in the prostate nucleus. It was shown, however, that like the nuclear receptor the soluble receptor is probably an acidic protein and that both cystein and tryptophan residues are necessary for maintaining the functional configuration of the receptor.

Liao and his co-workers (70) also found that following the injection of ^{3}H-testosterone into castrated rats the cytosol fraction of rat ventral prostate homogenates contained a specific 5α-dihydrotestosterone-binding protein which has a sedimentation coefficient of 3.5 S, while the dihydrotestosterone-bound protein extracted from the labeled nuclei by a buffered salt solution possesses a sedimentation coefficient of 3.0 S. Moreover, it was demonstrated in the in vitro experiments that if prostatic cell nuclei are first isolated from castrated rats and then incubated with ^{3}H-testosterone or ^{3}H-5α-dihydrotestosterone, there is no retention of dihydro-

testosterone in a protein-bound form; but upon addition of a cytosol fraction the nuclear fraction reisolated was found to retain ^{3}H-dihydrotestosterone as a complex with a 3 S protein. In vivo the rate of disappearance of the cytoplasmic 3.5 S dihydrotestosterone-protein complex is significantly more rapid than that of nuclear 3 S dihydrotestosterone-protein complex, suggesting a possible involvement of cytoplasmic factor(s) during the formation of the nuclear 3 S dihydrotestosterone-protein complex, or the retention of such a complex by nuclei. In addition, with many preparations the cytosol testosterone-protein complex migrates more slowly (2.7 S) than the dihydrotestosterone-bound protein, which indicates that more than one protein binds to different species of steroids with different affinity. The specific 5α-dihydrotestosterone-binding protein which is soluble in cytosol and the one bound firmly to the nuclei of rat ventral prostate have similar sedimentation coefficients which cannot be distinguished easily (70). However, these cytosol and nuclear proteins exhibit different heat sensitivity. The 3 S protein of the nuclear extract loses dihydrotestosterone-binding ability rapidly during incubation, with the result that more radioactive dihydrotestosterone remains at the top of the centrifuge tubes as a free steroid. On the other hand, the cytosol 3.5 S protein picks up considerably more ^{3}H-dihydrotestosterone during the incubation. The differential heat stability appeared to be the result of inherent properties of these proteins rather than of difference in the amount of other factors, such as proteolytic enzymes in the two protein fractions. If the dihydrotestosterone-bound proteins in the prostatic cell

nuclei and cytosol are indeed different, these observations are in line with the suggestion that 5α-dihydrotestosterone is first bound to a cytosol protein that is subsequently transferred to the nuclear binding sites. Since it has also been observed that in the absence of dihydrotestosterone prostatic cell nuclei do not retain any 3 or 3.5 S cytosol protein that would bind dihydrotestosterone spontaneously, this strongly suggests that prostatic cell nuclei have specific sites with a high affinity for a modified cytosol dihydrotestosterone-protein complex, but not for the original cytosol protein moiety alone.

Fang and Liao (92) found that in the rat ventral prostate there were at least two soluble cytoplasmic proteins which specifically bound ^{3}H-5α-dihydrotestosterone and that only one of the ^{3}H-dihydrotestosterone-complexes (Complex II) could be retained by cell nuclei of the same tissue. The other dihydrotestosterone-binding protein (Complex I) can be separated from Complex II by an ammonium sulfate fractionation and a Sephadex gel filtration and Complex I preparations strongly inhibit the retention of Complex II by the prostatic cell nuclei. It has also been found that Complex II, by itself, is not retained firmly by prostatic cell nuclei in the absence of 5α-dihydrotestosterone, suggesting that dihydrotestosterone 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-dihydrotestosterone, testosterone, 5α-androstane-3,17-dione, 5α-androstane-3α(or 3β),17β-diol, androst-4-ene-3,17-dione, 17β-estradiol, cortisol, and progesterone are not effective

for this purpose. The steroid- and tissue-specificity of the cell-free systems, therefore, strongly suggests that 5α-dihydrotestosterone plays a central role in the retention of an androgen receptor protein by prostate nuclei *in vivo*. The biological importance of the process is also underlined by the fact that cyproterone acetate, a potent anti-androgen, also inhibits the retention process in the cell-free system. These observations have led to the suggestion that a part of the steroid molecule is physically enveloped in the cytosol receptor protein molecule and that a part of the receptor-bound steroid also interacts with the nuclear acceptor and thus the steroid hormone acts "like glue" between the androgen receptor and the nuclear acceptor. If this is so, the hormone enfolded by two large macromolecules may not directly participate in the further biological function that may be triggered by the formation of the receptor-dihydrotestosterone-nuclear acceptor complex.

Unhjem's group (90,93) also studied androgen-macromolecular complex from the rat ventral prostate. Following administration of ^{3}H-testosterone to castrated rats or incubation of prostate tissue with the same steroid, 5α-dihydrotestosterone was the major component associated with the macromolecules isolated by a gel filtration technique both *in vivo* and *in vitro* experiments (90). The complex was destroyed by proteolytic enzymes like trypsin and pronase, but was unaffected by DNase and RNase. By sucrose density gradient centrifugation, two macromolecule components in high speed supernatants of Tris-HCl extracts from rat ventral prostate were found to be associated with radioactivity. By comparing the sedimentation

behavior of the two complexes with that of bovine serum albumin and beef liver catalase, the sedimentation coefficients of the complexes were calculated to be 9.3 S and 4.5 S. Unhjem (93) observed some structural requirements of steroids for binding to the macromolecules based on the results obtained from the in vitro experiments and found that an androstane skeleton with the 5α-configuration was essential for binding as well as a keto group at C-3. Under the same conditions 5α-androstane-3α,17β-diol (androstanediol) did not form complexes with macromolecules. The experiments carried out with the SH-reagents, N-ethylmaleimide, Na-iodoacetate, and p-hydroxymercuribenzoate, showed that these reagents effectively inhibited the binding of androgen to the macromolecules.

In the in vitro incubation experiments with a homogenate of the rat ventral prostate, the results obtained by Baulieu and Jung (94) are similar to these obtained by Unhjem et al. (93). On incubation of cytosol with ^{3}H-testosterone, 5α-dihydrotestosterone gave a heavy labeled complex, maximum in the 8-9 S region, and a lighter radioactive peak sedimenting in the same area as albumin (sedimentation coefficient 4-5 S). Radioactive 3α- and 3β-androstanediols were neither bound to the 8-9 S region nor to aggregates; they were largely bound in the 4-5 S region. Moreover, the 8-9 S binding had limited binding capacity, contrary to the case of the 4-5 S material. For example, dilution of radioactive ^{3}H-dihydrotestosterone 5×10^{-10} M with non-radioactive dihydrotestosterone 1×10 M decreased the radioactive 8-9 S peak by more than 80%, whereas this was not the case in the 4-5 S region. Specificity of the 8-9 S was certainly in favor of 5α-

dihydrotestosterone binding, but testosterone and to a much lesser degree 17β-estradiol could be bound. Other metabolites of testosterone were not bound at all. The 4-5 S binding had no limitation at hormone concentrations compatible with a physiological situation. There was no binding specificity in the 4-5 S region, and its sedimentation coefficient was equal to that of albumin.

In other in vitro cell-free experiments, Jung and Baulieu (95) analyzed the relationship between the steroid binding specificity (dihydrotestosterone versus testosterone) of the receptor system and the formation of nuclear receptor. In the rat ventral prostate, their results indicate two different binding systems and it was found that the dihydrotestosterone receptor had a sedimentation coefficient of 8-10 S. Testosterone was also bound to the 8-10 S fraction with smaller affinity, and to the same binding sites as dihydrotestosterone according to the results of competition experiments. On the contrary, binding of steroids in the 4-5 S region did not show steroid specificity. It was also observed that a specific dihydrotestosterone-binding protein in the cytosol acquired new properties when bound to dihydrotestosterone and was able to attach to some nuclear acceptor. Jung and Baulieu (95) have discussed some problems involved in this sequence of events as follows: There is only transformation of 20% of the cytosol receptor or dihydrotestosterone complex into the "neo-nuclear" form in cell-free experiments with a 1 : 1 cytosol to nuclear ratio on the basis of the weight of the prostates. The nuclear receptor has a sedimentation coefficient of 3 S, whereas the sedimentation coefficient of the cytosol receptor

in the same 0.5 M KCl solution is 4-5 S (94). It is possible that some proteolysis does occur in these in vitro experiments. Indeed, in the in vitro experiments, proteolysis may make some interpretations difficult; for example, the fact that testosterone does not lead to the formation of the neo-nuclear receptor can be eventually due to an insufficient protection of the cytosol binding protein by the relatively low affinity ligand. It is also possible to argue that the cytosol receptor may well be nuclear, partially at least, in the intact cell and artificially extracted during the homogenization of the tissue.

However, the studies made by Jung and Baulieu (95) indicate that, whether or not there is translocation of the dihydrotestosterone receptor complex, dihydrotestosterone and not testosterone provokes specific change(s) of the cytosol androgen receptor which enables it to attach to some nuclear acceptor (or more exactly, to some cellular structure sedimenting with the nuclear fraction, according to the authors).

In observations on the mechanism of testosterone action at the cellular levels by Steggles et al. (96), the preferential binding of dihydrotestosterone-prostate cytosol receptor to prostate chromatin, of estradiol-uterine cytosol receptor to uterine chromatin, and of progesterone-oviduct cytosol receptor to oviduct chromatin indicates that the chromatins of target cells have acceptor sites for specific steroid hormone-receptor complexes. For the ^{3}H-steroid to enter and be retained by the nucleus, two requirements must be met; first, the steroid must be associated with a specific cytosol receptor, and secondly, the nuclei must be from a specific

target tissue for that steroid. Steggles et al. (96) suggest that since extensive binding of specific cytosol receptor-steroid complexes occurs only with target tissue chromatins and is observed for dihydrotestosterone and estradiol as well as for progesterone, it may represent a general mechanism of action for steroid hormones in target tissue cells. After this nuclear event has occurred it is likely, but unproven, that steroid-mediated alterations in nuclear RNA metabolism and finally cytoplasmic protein synthesis occur.

Finally, in the in vitro and in vivo studies on the functional significance of androgen receptors in rat prostate, Rennie and Bruchovsky (97) found that ^{3}H-testosterone and ^{3}H-dihydrotestosterone became associated with a specific cytosol protein and then a specific nuclear protein. According to the authors, the receptors are closely related, but since they may not be identical it remains possible that testosterone and dihydrotestosterone are transferred from one receptor to the other, or alternatively the cytosol receptor may be modified slightly during its progression into the nucleus. It has been shown that no appreciable difference is noted in binding of ^{3}H-dihydrotestosterone to cytosol at 25^{o} or 37^{o}, contrary to some reports (70,93). It was not possible to saturate the cytosol receptor sites for either ^{3}H-testosterone or ^{3}H-dihydrotestosterone in the concentration range up to 10^{-7} M, again contrary to the report by Baulieu and Jung (94). Conversely, the nuclear binding of the two androgens reached an apparent plateau at approximately 5×10^{-8} M. Thus the binding reactions for both hormones exhibited qualitative similarities. On the basis of the

results obtained from the in vitro experiments, it has been found that there are 1,700 specific binding sites per nucleus for testosterone and 3,400 for dihydrotestosterone. Those values are somewhat different from Liao's report (70).

Although a number of reports (70,89,92,94,95,96) indicate that nuclear androgen binding occurs only in the presence of cytosol factor(s), Unhjem (91) has shown that cytosol factor(s) are not critical. The results obtained by Rennie and Bruchovsky (97) also indicate that significant binding takes place when nuclei are incubated in the presence of buffer.

Since it is expected that if 5α-dihydrotestosterone and testosterone were bound to the same sites on a single receptor protein, any shift in the binding of dihydrotestosterone caused by castration would be accompanied by a parallel shift in the binding of testosterone, Rennie and Bruchovsky (97) have compared the effects of castration on nuclear and cytosol binding in order to determine whether or not the receptor sites for both hormones are identical. The specific change in the binding of ^{3}H-dihydrotestosterone in response to castration was observed, whereas no corresponding changes observed in the binding of ^{3}H-testosterone. The results, therefore, suggest that the receptors for testosterone are not the same as those for 5α-dihydrotestosterone or that the experimental conditions to demonstrate the in vitro binding of each hormone are different.

It seems that the androgen receptors do exist which are involved in elicitin the biological response in the rat ventral prostate. However, since there are still a number of conflicting views on the mechanism

of androgen receptors at the molecular levels, further studies on these problems are needed.

Experimental Section

Steroids. 17α-^{14}C-Methyl-5α-androstan-17β-ol (^{14}C-12) and 17α-^{14}C-methyl-5α-androst-2-en-17β-ol (^{14}C-14) were obtained by a Grignard reaction of 5α-androstan-17-one and 5α-androst-2-en-17-one, respectively, with $^{14}CH_3MgI$. To $^{14}CH_3I$ (total 4 mc, specific activity 5 mc/mM; International Chemical & Nuclear Corp., Lot 554558) 1.82 ml of unlabeled CH_3I was added. The resulting CH_3I (total 30 mM) was then added to a stirred solution of 1.08 g Mg (45 mM) in 30 ml of ether (Et_2O) over a 45 min period. The mixture was refluxed for 1.5 hr. A solution of 2 mM steroid (5α-androstan-17-one: 548 mg, 5α-androst-2-en-17-one: 544 mg) in 30 ml of anhydrous benzene was added to 12 ml of the Grignard solution prepared above. The solvent was distilled off until the boiling point reached 78° and refluxing was continued for 5 hr. After cooling the mixture, 10 ml of 3 M CH_3MgBr (Alfa Inorganics) in Et_2O was added, the solvent was again distilled until the boiling point reached 78° and then the reaction mixture was refluxed for 3 hr. After the reaction mixture was stirred overnight at room temperature, a solution of NH_4Cl (1.4 g) in 25 ml of H_2O was added dropwise with stirring. The aqueous phase was extracted alternately with 30 ml of $CHCl_3$ and 20 ml of ethyl acatate. The combined extract and benzene phase were washed with dil HCl (1:3), 5% $NaHCO_3$ and H_2O. The organic phase was dried over anhydrous sodium sulfate and evaporated under reduced pressure to afford the crude products 480 mg of ^{14}C-12 and 460 mg of ^{14}C-14, respec-

tively. Recrystallization twice from acetone gave 230 mg of ^{14}C-12 and 200 mg of ^{14}C-14. Further purification and chemical purity check of these radioactive products were carried out by preparative layer chromatography and thin layer chromatography (benzene-acetone 5:1) to obtain radiochemically pure ^{14}C-12 and ^{14}C-14. The specific activities of ^{14}C-12 and ^{14}C-14 were 7.38 x 10^5 dpm/mg (90.9 μg/mM) and 8.12 x 10^5 dpm/mg (99.5 μc/mM), respectively.

17β-Hydroxy-1,2-^{3}H-5α-androstan-3-one (^{3}H-2) was purchased from New England Nuclear Corp., specific activity: 44 c/mM, Lot 635-068.

Cyproterone (6-chloro-17α-hydroxy-1α,2α-methylenepregna-4,6-diene-3,20-dione) was a gift from Dr. U. Kerb, Schering A-G, Berlin, whom the author thanks.

Dexamethasone (9α-fluoro-11β,17α,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione) was a gift from Dr. Ralph F. Hirschmann, Merck Sharpe & Dohme, Rahway, N.J., whom the author thanks.

Non-radioactive 17α-methyl-5α-androstan-17β-ol 12, 17α-methyl-5α-androstane-3α,17β-diol 16, 17α-methyl-5α-androstane-3β,17β-diol 17, and 17β-hydroxy-17α-methyl-5α-androstan-3-one 15 were prepared as described in the Part I experimental section.

17α-Methyl-5α-androst-2-en-17β-ol 14 was prepared by treatment of 5α-androst-2-en-17-one in anhydrous benzene with CH_3MgBr. The procedures were essentially the same as described above in the preparation of ^{14}C-14.

In vivo Experiments.

Male New Zealand White rabbits (4 kg) were used throughout the entire investigation.

One hr before sacrifice the rabbits were given 10 mg of radioactive steroid (^{14}C-12 or ^{14}C-14) dissolved in a saline solution containing 15 to 20% C_2H_5OH by deep intramuscular injection into the thigh. In another experiment, rabbits were given the radioactive steroids orally by feeding standard laboratory food (Purina Rabbit Chow) to which 30 to 40 mg of steroid (^{14}C-12 or ^{14}C-14) dissolved in C_2H_5OH was applied with a syringe. The animals were sacrificed at varying time intervals (1, 2, 4, and 8 hr) following the oral administration.

The ventral prostates were then dissected free of fat and adhering connective tissues, rinsed with saline solution, weighed, chopped with scissors, kept in a saline-acetone solution and left in a refrigerator overnight. Care was taken to exclude possible contamination with radioactive urine and blood. After evaporating acetone under reduced pressure, the aqueous phase was extracted with 4 x 15 ml ether. The ether extract was evaporated near dryness and transferred to a 10 ml volumetric flask with $CHCl_3$:C_2H_5OH (2:1). An aliquot of the solution was subjected to scintillation spectrometry to determine the total radioactivity recovered from the ventral prostate. The volume of the rest of solution was reduced under a stream of N_2 and the concentrated solution was subjected to tlc or glc to determine the radioactive content of metabolites originating from the labeled steroid administered.

In vitro Experiments.

Ventral prostate were excised from intact rabbits and minces of the prostatic tissue were suspended in Krebs-Ringer phosphate buffer (pH 7.4) containing radioactive steroids and incubated under an atmosphere of air for various intervals (15 min to 2 hr) at 37° in a shaking bath. Each incubation contained approximately 100 mg of the prostate minces in 4 ml of Krebs-Ringer solution containing the appropriate amount of radioactive steroid dissolved in C_2H_5OH. Control samples were prepared in a medium containing the same volume of C_2H_5OH (25-200 µl). The amounts of the labeled steroids incubated were 0.005 µg for ^{3}H-2 and 10 to 200 µg for ^{14}C-12 and ^{14}C-14.

In the competitive binding experiments, 25 to 300 µg of the appropriate non-radioactive steroid was added to the incubation mixture.

After the incubation was terminated, the prostate tissue was blotted with filter paper and washed in 5 ml of steroid-free Krebs-Ringer solution for 15 min to 2 hr at 37° in a shaking bath. The tissue was blotted, dried on filter paper to remove all moisture, transferred to weighed scintillation vials and weighed. The weight of the tissue was obtained by difference. To each vial 3 ml of $CHCl_3$:CH_3OH (2:1) was added and the vial was left in a refrigerator overnight to extract radioactive components. The solvent was evaporated to dryness under N_2 or air flow and the sample was subjected to scintillation spectrometry to determine radioactivity.

Liquid scintillation counting.

The radioactivity in each sample was counted in a Packard Tri-carb liquid scintillation spectrometer, Model 3375. Samples were deposited in nylon scintillation vials, and to each scintillation vial was added 14 ml of scintillation liquid consisting of 5 g of PPO (2,5-diphenyloxazole) and 100mg of POPOP (1,4-bis[2-(5-phenyloxazolyl)] benzene) in 1 ℓ of scintillation grade toluene. Quenching due to the prostate tissue and trace amounts of organic solvent was corrected by external standard ratio method. The counting efficiency of this system ranged from 26 to 32% for ^{3}H and 88 to 91% for ^{14}C. The identification of radioactive components were carried out by thin layer chromotography and gas liquid chromotography.

Characterization of radioactive steroid.

Separation of the labeled steroids in question were achieved on 6 cm x 20 cm silica gel tlc plates (2 mm thick, Brinkman) along with authentic carrier steroids. The plate was developed with benzene:$CHCl_3$:acetone (80:15:5). After the development, the plate was sprayed with ceric ammonium sulfate reagent consisting of 40 g of ceric ammonium sulfate and 70 ml of conc H_2SO_4 in enough water to make 400ml, and heated at 150° for 10 to 15 min. Each of the spraying reagent positive zones corresponding to the authentic standards which had been added to radioactive samples and the migration path on the plate were marked and divided into horizontal bands. Each band was scraped and placed into a scintillation vail, 14 ml of

scintillation liquid was added to the vial, and the sample was assayed for radioactivity. External standardization was used to correct for quenching with silica gel in a counting vial. A Varian Aerograph Series 2100 gas chromatograph equipped with an ionization detector and a 1:1 glass splitter was used. A 1.8 m glass column (0.8 cm inside diameter) was packed with 3% OV-1 on 100-200 mesh Gas Chrom Q. Conditions employed were: colum temperature 205°; injector 250°; detector 300°; He 30 ml/min; air 2.8 kg/cm2; and H_2 1.4 kg/cm^2. The vapors eluted from the column were condensed in capillary tubes by use of a splitter, and each sample for radioactivity determination trapped from the effluents was washed into a counting vial containing 14 ml of scintillation fluid.

Results

In order to see if significant amounts of metabolic products, especially the C-3 oxygenated derivatives, of 17α-methyl-5α-androstan-17β-ol 12 and 17α-methyl-5α-androst-2-en-17β-ol 14 are accumulated in the rabbit ventral prostate *in vivo*, ^{14}C-12 and ^{14}C-14 were administered by intramuscular injection into the thigh. In the rabbits given ^{14}C-12 or ^{14}C-14 by intramuscular injection, 70% - 80% of the radioactivity recovered from the ventral prostate was due to the unchanged parent compounds as can be seen from Table 3.

Following the oral administration of ^{14}C-12, however, approximately only half of the radioactivity recovered from the prostate was due to the original compound at varying time intervals studied. The results are presented in Table 4. In the rabbit administered ^{14}C-14 orally, on the other hand, virtually all radioactivity from the prostate resulted from the unchanged compound given.

The ability of rabbit ventral prostate to metabolize 12 and 14 *in vitro* were examined under the same conditions employed for the binding study. There was no significant amount of radioactivity found other than ^{14}C-12 or ^{14}C-14 after the incubation of minced prostate with those labeled steroids for 2 hr at 37^{o}.

The time sequence of the uptake and retention of ^{14}C-12 in the *in vitro* binding experiments was studied by incubating the minced rabbit prostate for 15 min to 2 hr at 37^{o}. The retention of radioactivity increased gradually during those periods of incubation as shown in Fig 9.

Table 3. Distribution of Radioactive Steroid Components in the Rabbit Ventral Prostate Following Intramuscular Injection of ^{14}C-Androgens.

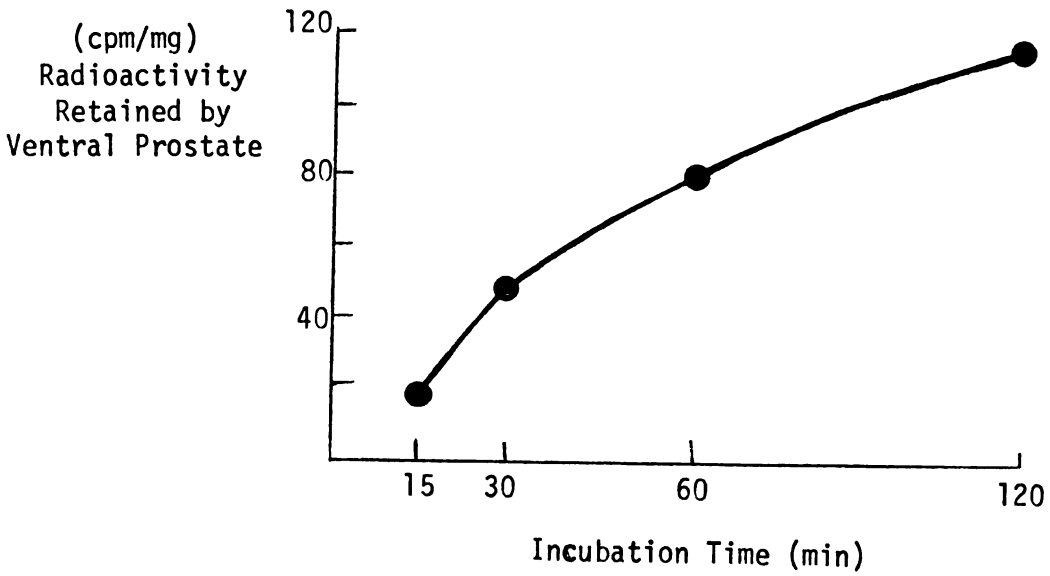
Compound Administered*	Total Radioactivity Recovered from Ventral Prostate (cpm/g tissue)	% of Radioactive Components in Ventral Prostate			
		Polar Metabolites	3-Hydroxy	3-Keto	Unchanged Parent Compound
17α-^{14}C-methyl-5α-androstan-17β-ol	2,006	9.1	14.4	6.7	69.8
17α-^{14}C-methyl-5α-androst-2-en-17β-ol	1,286	8.8	2.0	6.7	82.5

* The amount of the radioactive steroids injected was 10 mg each.

Table 4. Distribution of Radioactive Steroid Components in the Rabbit Ventral Prostate Following Oral Administration of ^{14}C-Androgens.

Time After Administration (hr)	Total Radioactivity Recovered from Ventral Prostate (cpm/g tissue)	% of Radiosctive Components in Ventral Prostate			
		Polar Metabolites	3-Hydoxy	3-Keto	Unchanged Parent Compound
17 -^{14}C-Methyl-5 -Androstan-17 -ol(40 mg for each rabbit)					
1	630	31.3	11.0	12.0	45.7
2	658	38.5	7.0	5.6	48.9
4	637	26.7	12.0	6.4	54.9
8	770	36.1	6.6	6.6	50.7
17 -^{14}C-Methyl-5 -androst-2-en-17 -ol (30 mg)					
6	926	0.3	0.2	0.3	99.2

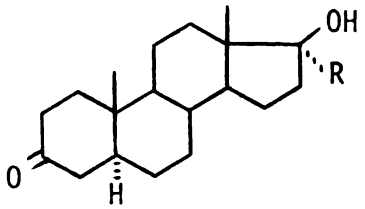
Fig 9. Time Sequence of the Uptake and Retention of 17α-^{14}C-Methyl-5α-androstan-17β-ol by Minced Prostate.



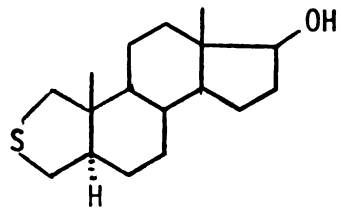
The uptake of radioactivity by minced rabbit ventral prostate at various time intervals, following incubation with 50 μg of 17α-^{14}C-methyl-5α-androstan-17β-ol at 37°. Each point on the curve represents the mean value of duplicate experiments.

The effects of concentration of ^{14}C-12 in the incubation medium and of washing time on the retention of radioactivity by the minced prostate were examined. Fig 10 shows that the amount of bound ^{14}C-12 in the prostatic tissue increased with increasing ^{14}C-12 concentrations in the incubation medium, approaching a limit at 100 µg and then remaining the same degree of labeling at 200 µg. After the first incubation of minced prostate with ^{14}C-12, the tissues were transferred to a hormone-free incubation medium in order to remove unspecifically adsorbed radioactive steroids. As shown in Fig 11, approximately 17% of the radioactivity was removed during the first 30 min period of washing and prolongation of the washing time up to 2 hr did not considerably alter the amount of radioactivity retained. In all subsequent in vitro competition experiments, the incubation was carried out for 2 hr and the time of washing in a steroid-free medium was 30 min at 37°.

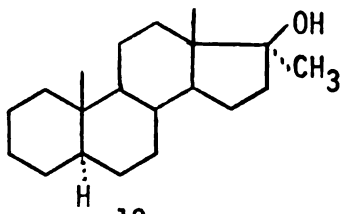
In the in vitro competitive uptake experiments, various unlabeled steroids were coincubated with each of three radioactively labeled androgens, 17α-^{14}C-methyl-5α-androstan-17β-ol ^{14}C-12, 17α-^{14}C methyl-5α-androst-2-en-17β-ol ^{14}C-14, and 1,2-^{3}H-17β-hydroxy-5α-androstan-3-one ^{3}H-2 (^{3}H-dihydrotestosterone), in order to determine if there are different binding sites for different steroids. Table 5 shows the percentage of radioactivity recovered from the prostate minces after incubation of ^{14}C-12 together with the unlabeled steroids listed. The retention of ^{14}C-12 was reduced by the presence of unlabeled steroids such as 17α-methyl-5α-androstan-17β-ol 12, 17α-methyl-5α-androstane-3α,17β-diol 16, and 17α-methyl-



2 R = H
15 R = CH_3



4



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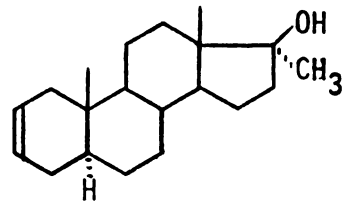

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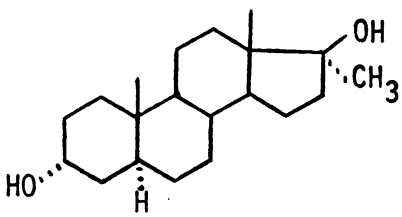

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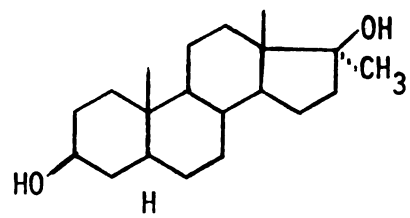

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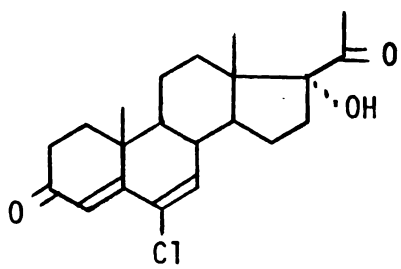
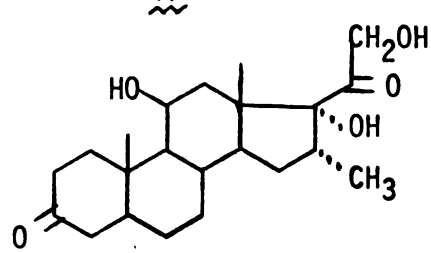
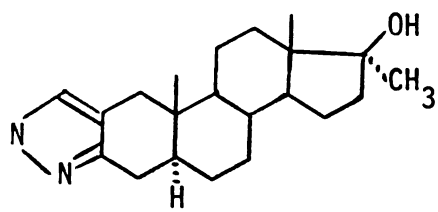


CYP

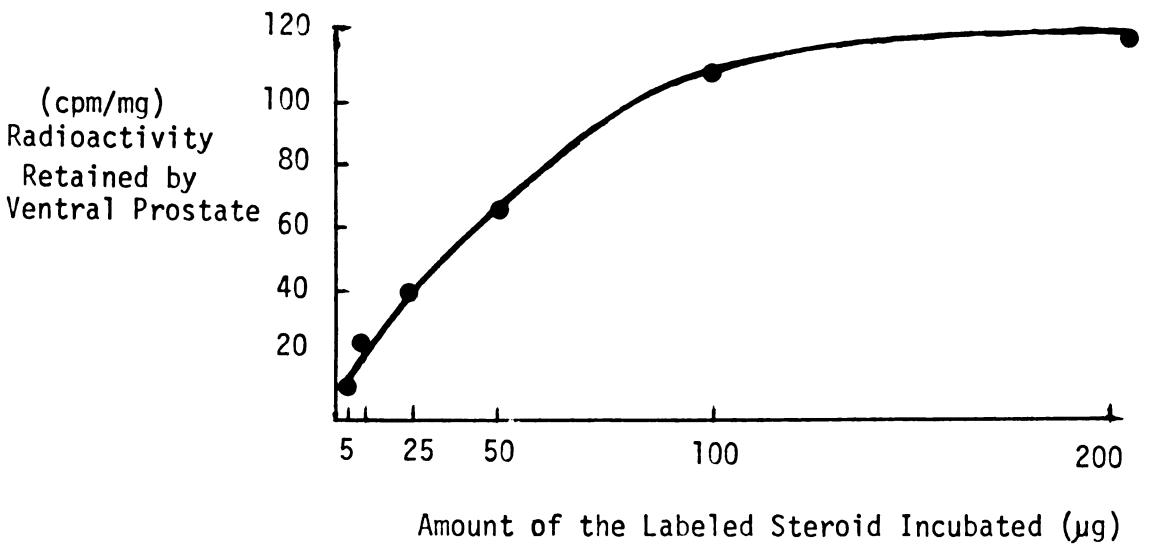


DEX



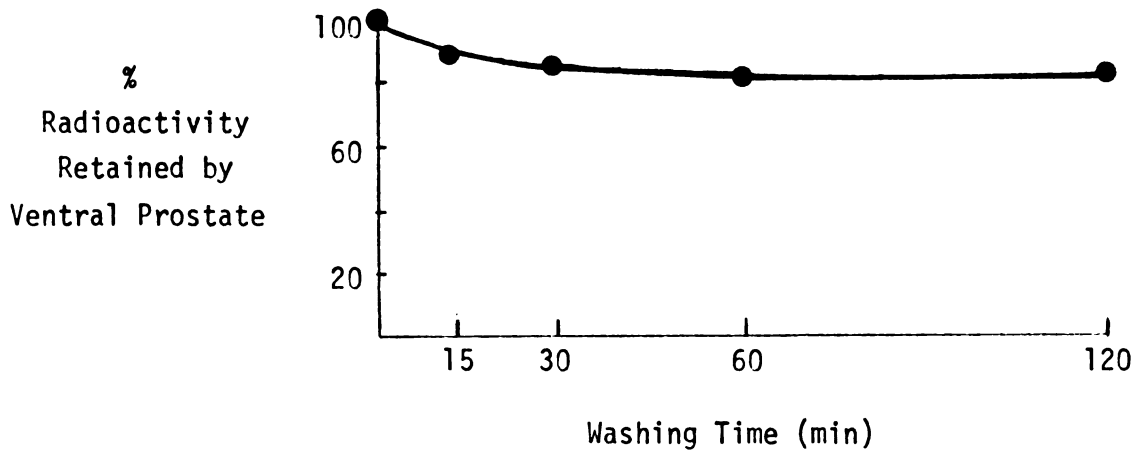
stanazolol

Fig 10. Effects of Concentration of 17α-^{14}C-Methyl-5α-androstan-17β-ol in Incubation Medium on the Retention of Radioactivity by Minced Prostate.



The retention of radioactivity by minced rabbit ventral prostate after the incubation of increasing amounts of 17α-^{14}C-methyl-5α-androstan-17β-ol for 2 hr at 37°, followed by washing in a hormone-free medium at 37° for 30 min.

Fig 11. Effects of Washing Time on the Retention of 17α-^{14}C-Methyl-5α-androstan-17β-ol by Minced Prostate.



The amount of radioactivity remaining in the minced ventral prostate in per cent of the controls at different time of washing in a hormone-free medium at 37°. Each point on the curve represents the mean value of duplicate experiments.

Table 5. Binding of 17α-^{14}C-Methyl-5α-androstan-17β-ol to Rabbit Prostate Minces.

Non-labeled Steroid Added	Radioactivity in the Minced Ventral Prostate (cpm/mg tissue)		% of Inhibition of Radioactivity Uptake
	Without Non-labeled Steroid (Control)	With Non-labeled Steroid (Competition)	
HC (12)	117.5 ± 10.3	52.2 ± 5.5	63.2
3α-OH (16)	119.8 ± 6.8	65.9 ± 3.2	45.0
3β-OH (17)	106.9 ± 9.2	60.4 ± 4.0	43.5
CYP	88.6 ± 5.2	90.4 ± 7.3	0
DEX	102.8 ± 10.7	97.1 ± 9.8	0
17α-Me-DHT (15)	93.3 ± 10.0	101.5 ± 10.4	0
2-ene (14)	110.4 ± 9.6	115.4 ± 11.2	0
2-thia (4)	93.5 ± 7.4	98.7 ± 5.0	0
stanazolol	100.7 ± 10.6	111.7 ± 9.4	0

For each set of competition experiment, the labeled steroid was coincubated with 200 μg of unlabeled steroid. The results are presented as cpm/mg wet tissue ± standard error for four determinations

5α-androstane-3β, 17β-diol 17. No reduction of the uptake of radioactivity as compared to the control was observed with other steroids tested.

When the uptake of minces incubated with ^{14}C-14 in competition with unlabeled steroids shown in Table 6 was investigated, only unlabed 14 or 2-thia-A-nor-5α-androstan-17β-ol 4 was found to reduce the uptake of radioactivity. No definite decrease in the retention of ^{14}C-14 was recorded with all of the other compounds examined.

As can be seen from Table 7, cyproterone (CYP), dexamethasone (DEX), and 17β-hydroxy-17α-methyl-5α-androstan-3-one 15 showed the blocking effect on the retention of ^{3}H-dihydrotestosterone by the minced prostate. In addition, the 2-thia compound 4 and stanazolol reduced the binding.

Table 6. Binding of 17α-^{14}C-Methyl-5α-androst-2-en-17β-ol to Rabbit Prostate Minces.

Non-labeled Steroid Added	Radioactivity in the Minced Ventral Prostate (cpm/mg tissue)		% of Inhibition of Radioactivity Uptake
	Without Non-labeled Steroid (Control)	With Non-labeled Steroid (Competition)	
2-ene (14)	108.0 ± 8.5	68.0 ± 5.0	57.8
2-thia (4)	116.5 ± 6.6	97.3 ± 4.3	16.5
3α-OH (16)	97.0 ± 7.6	94.4 ± 6.1	0
3β-OH (17)	102.4 ± 8.1	106.0 ± 6.8	0
HC (12)	112.6 ± 6.4	109.2 ± 10.4	0
17α-Me-DHT (15)	100.2 ± 9.8	98.8 ± 5.4	0
CYP	91.7 ± 8.4	93.7 ± 6.2	0
DEX	89.2 ± 6.3	82.5 ± 8.0	0
stanazolol	127.1 ± 10.7	120.4 ± 9.1	0

For each set of competition experiment, the labeled steroid was coincubated with 200 μg of unlabeled steroid. The results are presented as cpm/mg wet tissue ± standard error for four determinations.

Table 7. Binding of 17β-Hydroxy-1,2-^{3}H-5α-androstan-3-one to Rabbit Prostate Minces.

Non-labeled Steroid Added	Radioactivity in the Minced Ventral Prostate (cpm/mg tissue)		% of Inhibition of Radioactivity Uptake
	Without Non-labeled Steroid (Control)	With Non-labeled Steroid (Competition)	
17α-Me-DHT (15)	1284.9 ± 68.6	875.0 ± 49.1	31.9
CYP	1075.7 ± 24.4	689.5 ± 40.3	35.9
DEX	1166.2 ± 75.6	939.8 ± 31.8	19.4
CYP + DEX*	1003.1 ± 56.0	623.1 ± 25.2	38.0
2-thia (4)	1183.3 ± 88.2	778.8 ± 36.7	25.8
stanazolol	1428.2 ± 135.6	1030.0 ± 58.2	27.9
3α-OH (16)	1015.5 ± 73.4	1026.3 ± 61.4	0
3β-OH (17)	972.4 ± 58.5	946.6 ± 44.2	0
HC (12)	1074.9 ± 106.4	1048.0 ± 50.5	0
2-ene (14)	1027.4 ± 34.2	1013.6 ± 48.7	0

* The amounts of CYP and DEX were 100 μg each.
For each set of competition experiments, the labeled steroid was coincubated with 200 μg of unlabeled steroid. The results are calculated as cpm/mg wet tissue ± standard error for four determination.

Discussion

The general subject of the mechanism of steroid action has been an area of intensive research during the past decade, and has been reviewed (see, for example, 102,108). Receptor proteins for androgens and the mode of action of androgens on gene transcription have been reviewed by Liao and Fang (16). Two series of discoveries have contributed recently to advancing our knowledge of the mechanism of androgen action in the rat ventral prostate. First, among the testosterone metabolites formed in this target organ, 5α-dihydrotestosterone is preferentially concentrated in nuclei following the intraveneous administration of ^{3}H-testosterone to rats (79,80,81,87). Secondly, high affinity, low capacity steroid binding macromolecules, proteins according to indirect evidence and called "receptors", were observed in the nucleus (70,80,81,89), in the cytosol (70,89,90,92-97), and in the chromatin (88,96). Since extensively purified prostatic nuclear or chromatin alone is unable to retain dihydrotestosterone, it became clear that additional factors from the outside the nucleus itself were implicated in the selective binding process. These have been subsequently identified as specific hormone binding proteins or receptors in the cytoplasmic or cell supernatant fraction of the target organ. Overall, the uptake of 5α-dihydrotestosterone in the prostate follows a two-step binding process in which the steroid is first bound to an extranuclear receptor and finally transported into the nucleus. If the mechanism of steroid hormone action proposed by Jensen et al. (107) on the basis of study on estrogen receptors and investigated further by

Giannopoulos and Gorski (109) is generally applicable, then in the case of androgens too, the steroid hormone action at the target site is best described as follows. Upon entrance into a target cell, the steroid binds to cytoplasmic protein receptor(s) and the resulting complex is transported to the nucleus where it interacts with nuclear receptor(s) and transcription processes are affected.

Despite much work in this area, it is still not clear whether testosterone and 5α-dihydrotestosterone have different or the same cytosol receptor sites and the site of testosterone binding in the nucleus is also unclear (88,97). However, it is apparent that there is more than one androgen receptor even at the cytosol receptor level. A study on the binding of various synthetic androgens to target organs would be helpful in determining the type of receptor involved in binding various androgens.

As discussed in Part I of this thesis, 17α-methyl-5α-androstan-17β-ol is metabolized extensively by rabbit liver homogenate to the 3-hydroxy and 3-ketone derivatives, whereas no significant metabolic change was observed for 17α-methyl-5α-androst-2-en-17β-ol under the experimental conditions studied. Moreover, the 3α-hydroxy derivative is metabolized to the 3-ketone. These findings are compatible with the possibility that the A-ring hydrocarbon and 3α-hydroxy androgens exert their effect through conversion to dihydrotestosterone derivatives. However, the results do not exclude the possibility that these compounds function directly as androgens. In order to explore this question, the in vitro distribution of the two radioactive androgens, 17α- ^{14}C-methyl-5α-androstan-17β-ol ^{14}C-12

and 17α-^{14}C-methyl-5α-androst-2-en-17β-ol ^{14}C-14, by the rabbit ventral prostate was examined in the first part of the present study.

According to the results obtained in the in vivo experiments, these compounds are accumulated in the ventral prostate of male rabbits against a concentration gradient with blood, after the administration of these steroids by the oral route or intramuscular injection into the thigh. The data shown in Tables 3 and 4 indicate that the uptake of 12 and 14 by the prostate is significantly more extensive when these androgens are injected intramuscularly than when administered orally. The present data also indicate that the majority of the radioactivity recovered from this target organ after the intramuscular or oral administration of these labeled androgens is associated with the unchanged parent compound (Tables 3 and 4). These data, however, shed little light on the question of the active form of such compounds.

In the second part of the present study, the in vitro binding of the three radioactive androgens, 17β-hydroxy-1,2-^{3}H-5α-androstan-3-one ^{3}H-2, 17α-^{14}C-methyl-5α-androstan-17β-ol ^{14}C-12, and 17$_{\alpha}$-^{14}C-methyl-5α-androst-2-en-17β-ol ^{14}C-14, to minced rabbit ventral prostate was investigated. Particular attention was given to determining whether the retention of these labeled steroids occurs at the same site or different binding site(s) in the prostate. In competitive uptake experiments, the effect of several other non-radioactive steroids, such as 17α-methyl-5α-androstane-3α,17β-diol 16, 17α-methyl-5α-androstane-3β,17β-diol 17, 17β-hydroxy-17α-methyl-5α-androstan-3-one 15, cyproterone (CYP), and dexamethasone (DEX)

on the retention of these labeled androgens by rabbit ventral prostate was also investigated.

Although a number of studies on the binding of androgens like dihydrotestosterone to specific fractions of cytosol and nuclear proteins have been carried out, the present study describes the binding of androgens to prostatic minces. It was thought to be important to begin with these highly heterogeneous preparations in order to avoid the possibility of missing important binding components. This could happen if binding to only a few prostatic components were studied. There is, moreover, evidence that binding to whole prostatic tissue is subject to the same factors as binding to nuclear components (65,70,81,92,94,100). Thus it has been shown that cyproterone acetate inhibits the formation of nuclear dihydrotestosterone protein complex during incubation of minced ventral prostate in vitro with ^{3}H-dihydrotestosterone(81).

During the initial phase of this work, the possibility of metabolism of ^{14}C-12 and ^{14}C-14 was examined by incubation of the minced prostate tissue with these labeled steroids for two hours at 37°. In neither case was there significant radioactivity found other than that of the parent compound when extracts of these minces were examined by thin layer chromatography. The effects of length of incubation and of amounts of ^{14}C-12 on the uptake and retention of radioactivity by the prostate were also examined. The degree of labeling was found to increase gradually during the incubation period of two hours (Fig 9) and saturation of the binding of this compound was obtained (Fig 10). Finally, the effect of washing on retained radioactivity was examined (Fig 11). Some of the retained radioactivity was

removed upon washing, and the amount increased with time. A plateau level of about 83% was reached between 30 and 60 minutes and as much as two hours of washing did not further decrease this value. These preliminary experiments, therefore, served to determine the amount of steroid to be incubated, the length of the incubation time and the amount of washing required in the subsequent in vitro competitive uptake experiments.

In Table 5, the binding of the 17α-^{14}C-methyl hydrocarbon ^{14}C-12 to minced prostate is recorded. The binding of the labeled material is reduced by the addition of the unlabeled steroid 12. Moreover, the addition of 3α-hydroxy compound 16 and 3β-hydroxy steroid 17 effect significant reductions in the binding of ^{14}C-12. It is noteworthy that neither cyproterone (CYP) nor dexamethasone (DEX) nor any of the other steroids examined affects the binding of 17α-^{14}C-methyl hydrocarbon ^{14}C-12. By contrast, in Table 6 are shown the results of studying the binding of 17α-^{14}C-methyl 2-ene compound ^{14}C-14 to the prostate tissue. In this case, only the addition of unlabeled 14 or the addition of the 2-thia compound 4 inhibits the binding. All of other compounds examined, including CYP and DEX, are without effect on the binding. Finally, in Table 7 is shown the effect of various compounds on the binding of ^{3}H-dihydrotestosterone ^{3}H-2 to the tissue. In this case, 17α-methyl dihydrotestosterone 15 as well as CYP and DEX reduce the binding. In addition, the 2-thia compound and stanazolol reduce the binding. The effect of DEX was examined since it has been shown

by Steinetz et al. (110) that CYP blocks the androgenic effect of androgen analogs whereas DEX blocks the anabolic effects in levator ani preparations. Although these authors were unable to find effects on ventral prostate preparations under these conditions, the present finding of several different binding sites in the minced ventral prostate preparations made it of interest to examine the action of these selective inhibitors.

An interesting pattern of inhibitory effects on the binding is observed. The data are explained most readily by an assumption of the presence of three different binding sites for the materials tested. One of these is the "classical" site for 5α-dihydrotestosterone (5α-DHT). This binding site binds dihydrotestosterone as well as the 17α-methyl analog 15. The inhibition of binding at this site by CYP has already been observed by Fang, Anderson and Liao (70) and Geller et al. (69), who found that CYP suppressed the uptake of radioactive androgens in vivo by the ventral prostate of rats. Belham and Neal (71) also reported that cyproterone acetate inhibits the retention of labeled metabolites of ^{3}H-testosterone by prostatic nuclei, both in vivo and in vitro. In a study on the steroid- and tissue-specificity of the cell-free system, Fang and Liao (92) found that the potent anti-androgen inhibits the retention process of dihydrotestosterone in this system. The authors also suggest that dihydrotestosterone plays a central role in the retention of an androgen receptor protein by prostatic nuclei in vivo. 17β-Estradiol, diethylstilbestrol, and progesterone, but not cortisol, were reported to suppress the reten-

tion of dihydrotestosterone by prostatic cell nuclei in vitro, but to a much lesser extent than cyproterone acetate (70).

The 2-ene binding site apparently does not interact with any of the steroids which interact with the 5α-DHT site with the exception of the 2-thia compound 4. This interaction with the 2-thia compound would be expected on the basis of isosteric theory. A third bindind site, the hydrocarbon site, does not interact with any of the substances which interact with either the 2-ene site or the 5_{α}-DHT site.

Whether these receptors are in the cytosol or nucleus, or both, and whether they are different sites on the same macromolecules or represent different macromolecules is, of course, still an open question.

It is now an accepted concept that receptors are likely to be proteins and that the binding proteins play a fundamental role in the action of androgens. In regard to the construction of theoretical models for receptors, the assumption has been made that compounds having the same activity as the natural hormone bind to the same site and exhibit affinities parallel to the intensity of their respective biological effects. It is, however, hard to understand why compounds which have androgenic activity do not always show the blocking effects on the uptake or retention of a potent natural androgen, 5α-dihydrotestosterone, if it is assumed that all androgens exert their hormonal action by binding to the same sites on a single receptor protein. This suggests that some compounds produce the same hormonal action not only by competing for the same hormonal binding sites but also by binding to other sites in the responsive organ.

On the basis of the results obtained in the present study, it is clear that different androgens are involved in binding at different sites. This must, of course, be considered in connection with the structure-function requirements for androgens.

The results also suggest future lines of research. One of these is to fractionate the cytosol proteins and to try to localize the binding sites more distinctively. Another is to study a wide range of androgens and their interaction with these sites. The results of such studies should give a better insight than we have at present regarding the structure-function relationships in androgen molecules.

SUMMARY

The possibility that 5α-androstane derivatives having a simple hydrocarbon A ring can be oxygenated metabolically to known C-3 oxygenated androgens was examined. The following 3-oxygenated androstanes were isolated and characterized by glc and mass spectrometry after incubation of 17α-methyl-5α-androstan-17β-ol 12 with rabbit liver homogenate: 17β-hydroxy-17α-methyl-5α-androstan-3-one 15, 17α-methyl-5α-androstane-3α,17β-diol 16, and 17α-methyl-5α-androstane-3β,17β-diol 17. The results obtained from a study of the metabolism of 15, 16, and 17 by rabbit liver homogenate show that 15 and 16 largely give the corresponding derivatives 16 and 17, and 15 and 17, respectively, whereas only a small conversion of 17 to 15 and 16 was observed.

The *in vivo* study of uptake of 17α-^{14}C-methyl-5α-androstan-17β-ol ^{14}C-12 and 17α-^{14}C-methyl-5α-androst-2-en-17β-ol ^{14}C-14 by rabbit ventral prostate showed that the majority of the radioactivity recovered from this target organ was associated with the unchanged parent compounds after the administration of ^{14}C-12 and ^{14}C-14 orally or intramuscularly.

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hydrocarbon and the 2-olefin derivatives. The implications of this for structure-activity relationships of androgen analogs have been discussed.

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