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CORRELATION OF *IN VIVO* AND *IN VITRO* ACTIVITIES OF SOME NATURALLY OCCURRING ANDROGENS USING A RADIORECEPTOR ASSAY FOR 5α-DIHYDROTESTOSTERONE WITH RAT PROSTATE CYTOSOL RECEPTOR PROTEIN

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SUMMARY

A radioreceptor assay for 5α-dihydrotestosterone (DHT) has been developed using rat prostate cytosol receptor protein. Characterization studies reveal that testosterone (T) also competes for the same binding sites as DHT. Estradiol; dehydroepiandrosterone; 4-androsten-3,17-dione; progesterone; cortisol and clomiphene citrate do not compete for binding. The binding of DHT to castrate rat prostate cytosol was greater than the same preparation from noncastrate rats and was maximal at 9–36 h after castration. This assay was used to assess potencies of naturally occurring androgenic steroids. There was little correlation between the known *in vivo* activity and the *in vitro* response of some of these naturally occurring androgens. This data, considered along with published data of others, indicate that the *in vivo* androgen activity of these steroids is due to metabolic conversion to an active metabolite.

INTRODUCTION

It has been shown that compounds structurally unrelated to estradiol show *in vivo* estrogenic and antiestrogenic activity[1]. Some of these also compete for the same binding sites as estradiol in an *in vitro* system using uterine cytosol receptor preparation[2]. This indicates the relative non-specificity of estrogen receptors; these receptors appear to recognize the aromatic π cloud only in certain orientation.

The structural requirements for steroids possessing androgenic activity *in vivo* appear to be much more specific; very few non-steroidal compounds have been shown to possess *in vivo* androgen activity[3]. Changes in the tetracyclic structure of T or DHT, either to disrupt the coplanarity of the molecule by changing the stereochemistry at the ring junctions, or substitution at different positions to cause molecular distortion, makes the new compounds devoid and/or lowers the *in vivo* androgen activity[3]. In addition, based upon the *in vivo* response of a large number of differently substituted androstanes and androstenes, several theories for androgen-receptor interaction have been proposed. Vida has reviewed all these theories[4].

Fang, Anderson and Liao[5] and Bruchovsky and Wilson[6] have shown that the active androgen for the prostate stimulation is not T, but DHT which is formed by metabolic conversion from T in the prostate. Anderson and Liao[7] also showed that DHT binds initially to the cytosol as a 3·5S complex which is then transferred to the nucleus as a 3S complex; and this, in turn, is responsible for the gene transcription in the nucleus. Emphasis was, however, laid on the initial binding of DHT to the cytosol.

It has been observed that *in vivo* some synthetic steroids belonging to the 19-nor T series are much more potent than T[8]. In a recent publication Liao *et al.*[9] have shown by using sucrose gradient ultracentrifugation technique that some of these compounds do indeed bind more strongly to the prostate cytosol than DHT and are transferred to the nuclei without metabolic conversion to the corresponding 19-nor DHT compounds. Interestingly, these compounds bypass the 5α-reductase step which is probably a prerequisite for the biological action of T in the prostate. One of the factors considered for the increased *in vivo* and *in vitro* activity of these compounds is the increased coplanarity of the tetracyclic structure of the steroid molecule[9].

Recently, Bruchovsky[10] studied the *in vivo* metabolic fate of some natural androgens in the prostate. The results indicate that although several metabolites are formed from each compound, invariably one of the major metabolites was DHT.

The following are the chemical names for the trivial names used in the text of this paper. 5α-Dihydrotestosterone (DHT) 17β-hydroxy-5α-androstane-3-one; testosterone (T), 17β-hydroxy-4-androsten-3-one; estradiol, 1,3,5(10)-estratriene-3,17β-diol; dehydroepiandrosterone, 3β-hydroxy-5-androsten-17-one; progesterone, 4-pregnene-3,20-dione; cortisol, 11β,17,21-trihydroxy-4-pregnene-3,20-dione; clomiphene citrate, 1[p-(diethylaminoethoxy)-phenyl]-1,2-diphenyl-2-chloroethane citrate.

Highest levels of nuclear radioactivity were achieved with T, DHT and 5α-androstane-3α,17β-diol; intermediate levels were obtained with androsten-dione, androstane-dione and androsterone and a low level with dehydroepiandrosterone. The *in vivo* metabolic fate of isomeric 4-androsten-3β,17β-diol and 5-androsten-3β,17β-diol has not been studied.

We, in this paper, wish to report a simple and sensitive *in vitro* assay for the measurement of DHT binding using castrate rat prostate cytosol receptor protein preparation. We have used this assay to assess the competitive displacement of labelled DHT by some androgens. The data seem to indicate that the *in vivo* activity associated with some of the androgens is due to their metabolic conversion to an active metabolite and not to their androgen properties *per se.*

MATERIALS AND METHODS

Radioactive steroids (40 Ci/mmol for [1,2-^{3}H]-DHT, 100 Ci/mmol for [1,2,6,7,-^{3}H]-T, and the scintillation fluid [Aquasol^R]) were obtained from New England Nuclear. Non-radioactive steroids used in this study were purchased from Steraloids, Inc., Pawling, New York. Clomiphene citrate was purified from Clomid tablets (The W. M. S. Merril Company, Cincinnati, Ohio) by extracting the powdered tablets with ethanol and crystallization of the residue left after evaporation of ethanol with a mixture of methanol and ethyl acetate to the required chemical purity.

Tris–HCl (10 mM), pH 7·4 containing 1·5 mM EDTA was used in all experiments and is referred to as Tris buffer.

Three hundred gram male Wistar rats were utilized for prostate source. Castration was performed *via* the scrotal route with the use of ether anesthesia. Animals were sacrificed using CO_2 narcosis and ventral prostates were removed, and placed in Tris buffer maintained at 4°C. The tissues were blotted, rapidly weighed on a torsion balance at 4°C and homogenized in Tris buffer (3 times v/v as the weight of the prostates) using all glass homogenizer at 4°C. The homogenate was centrifuged at 100,000g for 1 h and the supernatant used for the assays. Using this method of preparation, only cytosol receptor proteins are obtained; intact nuclei and nuclear and cell membranes are found in the pellet after centrifugation.

RESULTS

Separation of receptor bound and free steroids

(a) To determine the proper amount of charcoal to be used to separate receptor-bound from free hormone, heated prostatic cytosol (to destroy any receptor proteins) supernatant was incubated with 10 pg of [^{3}H]-T. The heat killed cytosol was added to maintain protein concentration constant, since the amount of charcoal selected must bind free hormone when active cytosol proteins were added in a series of tubes. Binoux and Odell have previously reviewed the details required to select the optimal amount of charcoal required to separate bound from free hormone[12]. Thus, varying amounts of 10% Dextran-coated charcoal, between 1 and 1000 μg/ml, were added and the tubes incubated for 10 min at 4°C. All tubes were centrifuged at 2000 rev./min and the supernatant taken up in 10 ml of aquasol for determination of radioactivity. A semilog dose-response curve of per cent counts bound to charcoal versus amount of charcoal added was constructed and is shown in Fig. 1a. The amount of charcoal required to bind

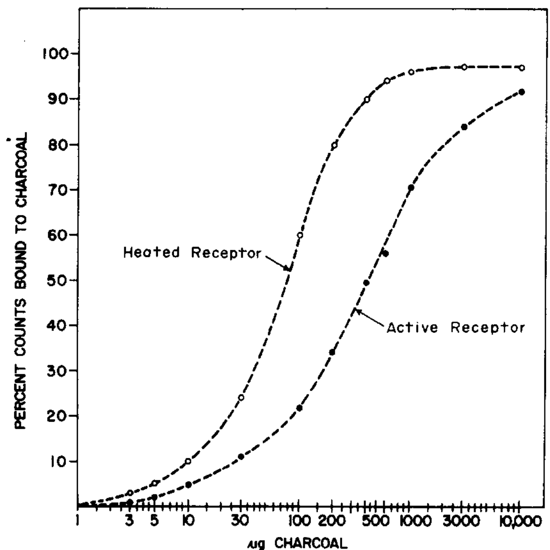

Fig. 1a. Dose response curves for 10% Dextran-coated charcoal binding of free DHT (heat killed receptor) and receptor bound DHT. For assay purposes 250 μg of charcoal was selected.

about 85% of the tritiated steroid was determined in this fashion. Then, using excess "living" cytosol receptor, we showed that this amount of charcoal bound some of the receptor bound DHT, but that most was not bound. Furthermore, in the range of charcoal selected, variation in charcoal added had little effect on the per cent counts bound in tubes containing excess receptor or no receptor, since the two curves were nearly parallel in this zone. In addition, the per cent DHT bound in the presence or absence of receptor did not vary with time of incubation with charcoal between 10 and 120 min. For all assays, 1000 μl of 250 μg/ml charcoal suspension coated with 10% Dextran was used to separate free from bound hormone. Analysis of our data reveals that charcoal is not an optimal material to separate bound from free hormone for this assay, but that it is usable. An optimal reagent would bind none of the receptor-bound and all of the free hormone. Such perfection, according to our systematic recent analysis[13] is seldom achieved. As we have recently shown[12] Dextran

coating changed only the amount of charcoal required, by blocking non-specific surface adsorption sites, without resulting in "molecular sieving." Stated in other words: the dose-response curve for charcoal was right shifted when compared to uncoated charcoal. In addition, varying protein concentrations also changed the amount of charcoal required. For these reasons, the amount of charcoal we have used would be incorrect to use in solutions containing less or more protein or if non-Dextran-coated charcoal were used.

(b) Similarly, to determine the proper concentration of polyethylene glycol to separate receptor bound from free hormone the following set of experiments was performed. Three series of tubes were incubated in varying final concentrations of polyethylene glycol—2% gamma globulin ranging from 0·5 to 12%. One series contained 3 pg of [^{3}H]-DHT, Tris buffer and castrate rat prostate cytosol receptor. A second series contained the same reagents plus excess non-radioactive DHT (1 μg). A third series contained only [^{3}H]-DHT. Fig. 1b shows that in the absence of non-radioactive DHT the binding increased from 7% to 35%, whereas in the presence of non-radioactive DHT the binding was 2·5% to 12%. Significantly, in the absence of live receptor and non-radioactive DHT there was no precipitation of free DHT at higher concentrations of polyethylene glycol as has been observed with other hormones in the antibody system [14]. The best final concentration of polyethylene glycol appeared to be 10% and this was selected to be used in further studies.

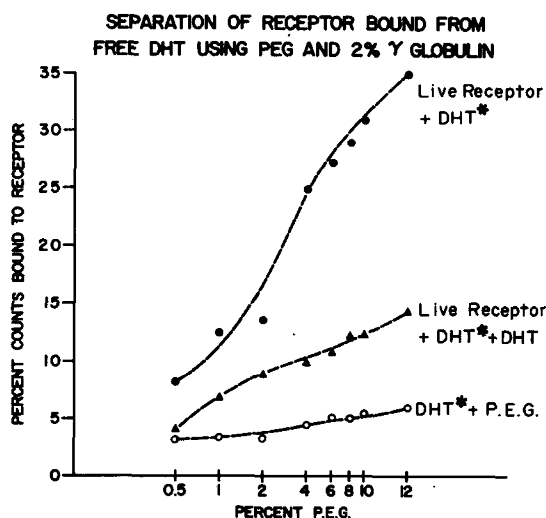

Fig. 1b. Dose response curves for polyethylene glycol (PEG) using live receptor and [^{3}H]-DHT (DHT*); live receptor, DHT* and excess unlabelled DHT (DHT* + DHT); and DHT* in the absence of receptor (DHT* + PEG).

Dilution of receptor preparation

The effects of various dilutions of receptor preparation were evaluated by adding serial dilutions of the receptor solution to constant amounts of [^{3}H]-DHT and determining the per cent of DHT specifically bound (displaceable by non-radioactive DHT). A dose-response curve was constructed and the data indicated that dilutions of greater than 3 vol. of buffer to 1 g of prostate gave less binding. Thus, a dilution of 1 g of prostate/3 ml buffer was utilized in all subsequent assays. More concentrated preparations were impractical.

Stability of the receptor preparation

The receptor preparation was kept for varying periods of time at several temperatures to determine stability for use. When kept at room temperature no specific binding could be observed after 24 h. Specific binding also decreased by 35% when the preparation was kept at 4°C for 24 h. However, when quick frozen in dry ice-alcohol and kept frozen for 24 h, the fall-off was only 10% of that of fresh preparation. Lyophilized preparation has not been studied; it seems more appropriate to estimate the number of animals required for a given assay and to utilize the entire cytosol preparation.

Effect of time after castration on binding

The effect of time after castration of rats on specific binding of DHT to cytosol receptor proteins was studied. In these studies cytosol was prepared by adding 3 ml of buffer to 1 g of prostatic tissue. For comparative purposes the specific binding of DHT present at 9–36 h was defined as 100% and all other times related to that. Specific binding was defined as counts bound and displaceable by 1 μg of non-radioactive DHT. Figure 2 shows that the per cent of maximal binding was zero in noncastrate animals and increased with time after castration. It reached a maximum at 9 h and stayed the same up to 36 h. After 36 h binding it fell to zero again by 144 h after castration. Presumably, at times before 20 h, change in binding was due to depletion of endogenous DHT from the binding sites on the receptors—while after 36 h the receptor population *per se* decreased.

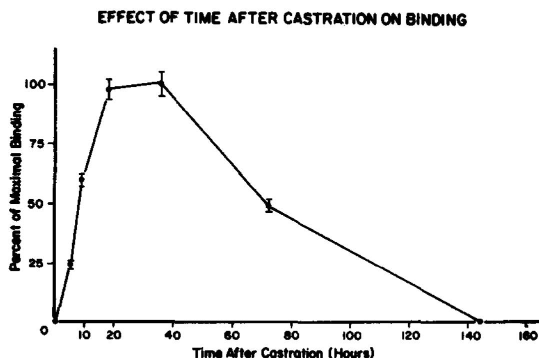

Fig. 2. Effect of time after castration on binding of DHT. Per cent of maximal binding, i.e. per cent of maximum counts displaceable by non-radioactive DHT at 9 h, is shown on the Y-axis. Each point on the curve represents the mean value of four animals. The weight of prostate tissue per vol. of diluent was constant throughout these studies. e.g. 1 g/3 ml.

Assay procedure

The following procedure was adopted for routine use. Adult male Wistar rats were castrated under ether anesthesia. Eighteen hours later they were sacrificed under carbon dioxide narcosis and the prostates immediately removed and placed in Tris buffer maintained at 4°C. All reagents were added in cold room at 4°C to 10 × 75 mm test tubes. Three picograms of [^{3}H]-DHT (or 1000 d.p.m.) were added to each tube. The non-radioactive steroids in proper dilutions (10 pg to 10 μg) were added in duplicate and the organic solvent evaporated under a slow stream of nitrogen. To each tube 400 μl of Tris buffer and 100 μl of the cytosol preparation were added. Each tube was stirred in a vortex stirrer and incubated at 4°C for 2 h. After incubation the separation was achieved by two methods. (a) 1 ml of the charcoal suspension (250 μg/ml) in Tris buffer containing 10% Dextran was added to each tube and the mixture stirred and permitted to stand at 4°C for 10 min. All tubes were centrifuged at 2000 rev./min in a refrigerated centrifuge. The supernatant was aspirated with a Pasteur pipette and added to 12 ml of aquasol. (b) 100 μl of 2% gamma globulin in Tris buffer and 600 μl of 20% polyethylene glycol in Tris buffer were added and the mixture stirred and allowed to stand at 4°C for 30 min. All tubes were centrifuged at 2000 rev./min in a refrigerated centrifuge. The precipitate obtained after aspiration of the supernatant was dissolved in water 1 ml and counted in 10 ml of aquasol.

Potency of naturally occurring steroids

Using the 18-h postcastration rats, detailed dose-response curves were analyzed. When [^{3}H]-DHT was added to the cytosol fraction of the prostatic tissue, protein binding occurred which was inhibited by non-radioactive DHT and also by non-radioactive T, 4-androsten-3β,17β-diol, 5-androsten-3β,17β-diol, 5α-androstane-3β,17β-diol, 5α-androstane-3α,17β-diol, and 5α-androstane-3,17-dione. However, binding was not inhibited in this assay system by up to 1 μg of non-radioactive estradiol, dehydroepiandrosterone, 4-androsten-3,17-dione, progesterone, cortisol, 3β-hydroxy-5α-androstan-17-one and clomiphene citrate. The dose-response curve using non-radioactive DHT, prostate cytosol receptor protein and [^{3}H]-DHT using charcoal is shown in Fig. 3a. Using 1000 d.p.m. of [^{3}H]-DHT as the binding steroid, non-radioactive DHT competed for the same binding sites as radioactive DHT. The dose-response curve existed between 10 and 300 pg of DHT. Figure 3a shows that T competed for the same binding sites and a parallel dose-response curve exists. However, in this assay system T is 33% as potent as DHT [15]. Figure 3b shows that the same results are obtained if polyethylene glycol is used to separate free from the bound hormone. Similar results were obtained using radioactive T.

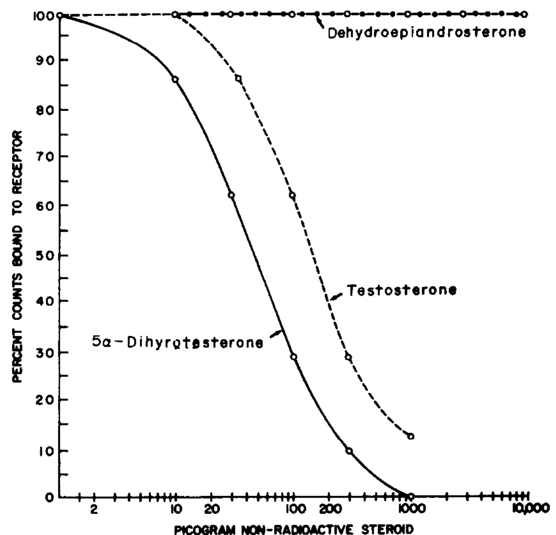

Fig. 3a. Dose response curves for DHT and T, and dehydroepiandrosterone in the receptor assay using ^{3}H-DHT and charcoal. The per cent counts bound is plotted on the Y-axis; 100% was defined as the maximum counts bound, zero per cent as the counts bound in the presence of excess unlabelled steroid (3000 pg). This permitted comparison of several assays. The actual per cent counts bound was approximately 50% (the 100% value), and approximately 30% (the zero per cent value). The rest was non-specific binding.

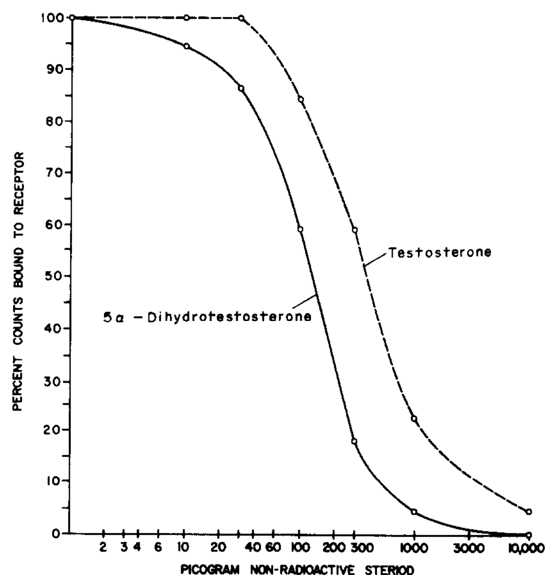

Fig. 3b. Dose response curves for DHT and T in the receptor assay using [^{3}H]-DHT and polyethylene glycol. The actual per cent counts bound was approximately 25–30% (the 100% value) and approximately 5% (the zero per cent value). The rest, 5%, was non-specific binding.

Figures 4 and 5a,b show the competitive displacement of DHT with various naturally occurring androgens using charcoal. The salient features of these curves being that while 5α-androstane-3α,17β-diol (Fig. 4) is a very potent androgen *in vivo*, it is a weak competitor for DHT in *in vitro* system. This is also true for 4-androstene-3β,17β-

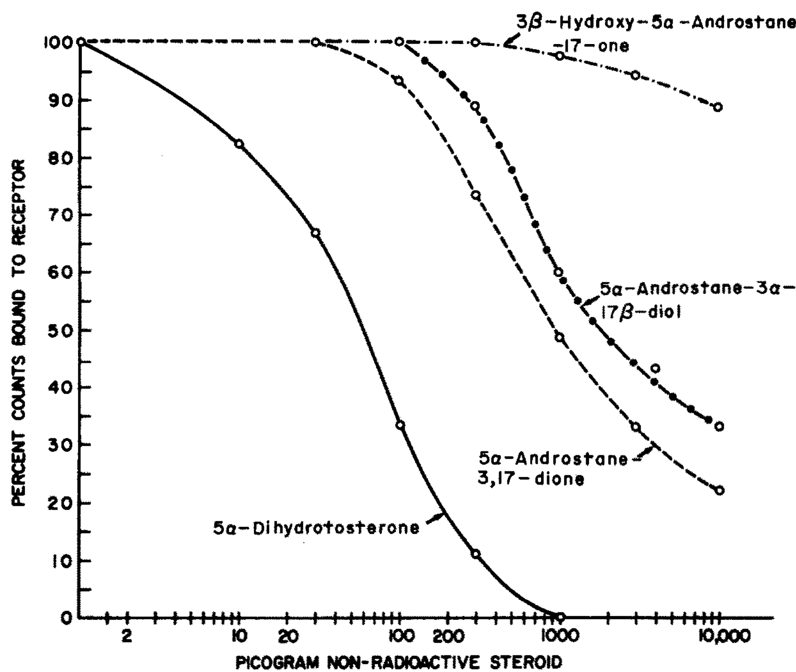

Fig. 4. Dose response curves for 5α-androstane-3,17-dione, 5α-androstane-3α,17β-diol and 3β-hydroxy-5α-androstane-17-one as compared to DHT.

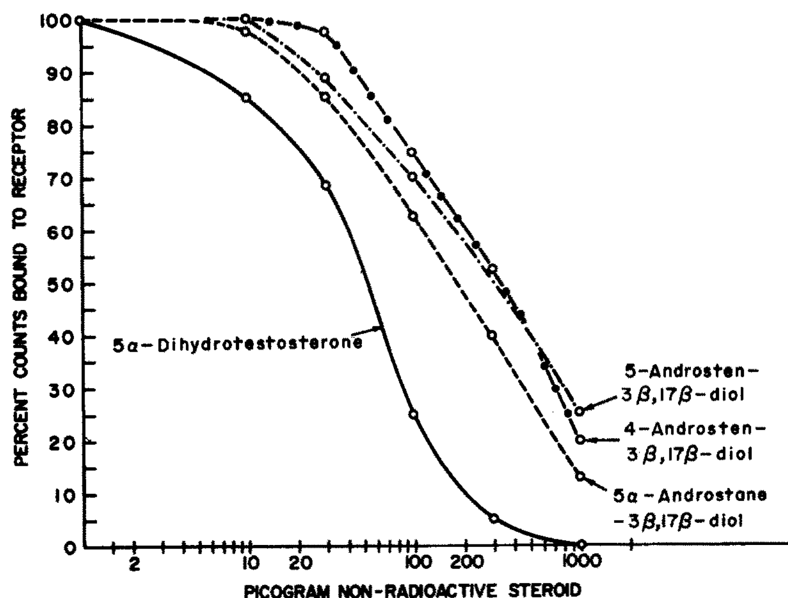

Fig. 5a. Dose response curves for 5α-androstane-3β,17β-diol, 4-androsten-3β,17β-diol and diol as compared to DHT using charcoal.

diol (Fig. 5a). Comparable results were obtained (Fig. 5a,b) when competitive displacement of DHT with 4-androstene-3β,17β-diol and 5-androstene-3β,17β-diol was studied using polyethylene glycol instead of charcoal to separate free and bound hormone.

DISCUSSION

Fang *et al.*[5] and Bruchovsky and Wilson[6], using sucrose gradient ultracentrifugation technique to separate receptor bound from free hormone, showed that non-radioactive DHT displaces bound DHT from the cytosol fraction of castrate rat prostate. By using a new and different *in vitro* assay system and employing two independent techniques to separate receptor bound from free hormone, we have demonstrated that the cytosol fraction of castrate prostate binds tritiated DHT which is displaced by non-radioactive DHT in a dose response relationship (Fig. 3a,b). However, in contrast to the findings of Anderson and Liao[7], we have observed that T and 5α-androstane-

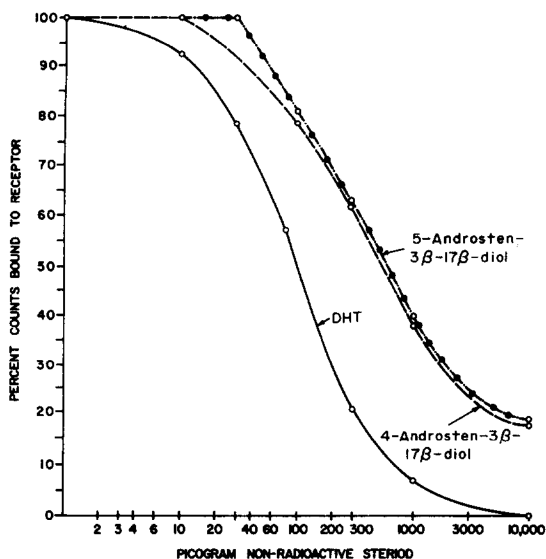

Fig. 5b. Dose response curves for 4-androsten-3β,17β-diol and 5-androsten-3β,17β-diol as compared to DHT using polyethylene glycol.

3β,17β-diol also binds to the same cytosol preparation and competitively displaced labelled DHT. Testosterone was only 33% as active as DHT, a potency that agrees relatively well with the *in vivo* activity of T and DHT. In a recent publication [16] Sullivan and Strott, using sucrose–ethylene glycol gradient ultracentrifugation technique have also shown that T is 33% active as compared to DHT. The competitive displacement of DHT with various naturally occurring androgens was studied and is summarized in Fig. 6. Four-androsten-3,17-dione and dehydroepiandrosterone are 15 and 12·6% respectively, as potent *in vivo* (DHT = 100%); however, studied *in vitro* receptor assay, they are inactive. Presumably the *in vivo* activity of androstene-dione results from metabolism to DHT [6, 7, 10]. Dehydroepiandrosterone, a precursor of androstene-dione, is the key intermediate in the biosynthesis of testosterone in the testes [11]. 3β-Hydroxy-5α-androstane-17-one is neither active *in vivo* or in the *in vitro* assay.

The presence of an oxygen function at C-3 and a 17β-hydroxyl group in steroids is necessary for the *in vitro* androgen activity. For an androgen to be active *in vitro* the presence of a 17β-hydroxy group seems to be very important and the oxygen function at C-3 can be in the form of a keto group or a hydroxyl group. *In vivo* and *in vitro*, the keto group at C-3 imparts more activity than the hydroxyl because its polarized form can effectively take part in non-bonded interactions with the receptor protein. While the 3-hydroxyl compounds are less active, the stereochemistry of the hydroxyl group seems to play an important role. It is known that steroids devoid of an oxygen function at C-3 show weak *in vivo* androgen activity [4], which is due to their metabolism to a metabolite carrying a 3-oxygen function. This was actually demonstrated

Steroid	In Vivo %	In Vitro %
1	100.0	100.0
2	37.0	33.0
3	15.0	not active
4	12.6	not active
5	46.0	12.7
6	7.4	12.7
7	12.2	6.6
8	0.74	not active
9	1.1	20.0
10	100.0	2.3

Fig. 6. Relative androgen potencies of 10 steroids *in vivo* (prostate weight assay) and *in vitro* (prostate cytosol assay). Potency of DHT was defined as 100%.

by Wolff *et al.* [17] that *in vivo* activity of 17α-methyl-17β-hydroxy-5α-androstane is due to its metabolic conversion to 17α-methyl DHT in rabbit liver.

Liao *et al.* [9] have shown that 19-nor T derivatives which are more potent androgens *in vivo*, also bind strongly to the prostatic cytosol receptors and are transferred to the nuclei without metabolic conversion of the corresponding 19-nor DHT derivatives thus bypassing the 5α-reductase step. Based on these results, Liao has postulated that the presence of extra double bonds in 19-nor T molecule make the whole molecule very flat and coplanar and thus can fit a narrow hole or M-site on the receptor better than T or DHT. In other words, the meaningful androgenicity of a compound would depend upon how coplanar the whole molecule is and thus would fit the M-site on the receptor.

The stereochemistry of substitutents at C-3 which can contribute to coplanarity or flatness of the molecule would be an important factor for the *in vitro* activity of isomeric androstanediols and androsten-diols. Since the 3β-hydroxyl in 5α-androstane-3β,17β-diol is equatorially oriented and essentially coplanar with the rest of the molecule, it would be expected to fit the binding site on the receptor better than the corresponding 3α isomer which is axially oriented and is out of plane with the whole molecule. We report herein that this is true; the 5α-androstane-3β,17β-diol was more active

(20%) in our *in vitro* assay than the 3α isomer (2·3%). Their reversed *in vivo* activity (1·1% and 100% respectively) can be rationalized from the fact that the axially oriented 3α hydroxyl group in steroids is known to be more readily oxidized chemically and biochemically to the corresponding 3-keto compound, than the 3β isomer in which the hydroxyl group is equatorially oriented[18].

The isomeric androstene diols (i.e.) 4-androsten-3β,17β-diol and 5-androsten-3β,17β-diol show the same activity *in vitro* (12·7%). Construction of the Dreiding models indicate that conformationally the two compounds are very similar and there is practically no distortion in the coplanarity of the molecule. The hydroxyl groups which are equatorially oriented fall in the plane of the molecule so they are expected to fit the receptor with similar affinity. Our *in vitro* studies showed this to be true. However, *in vivo* 4-androsten-3β,17β-diol is a more potent androgen than the corresponding isomer 5-androsten-3β,17β-diol (46·0% and 7·4% respectively).

The biosynthesis of T in the testes has been extensively studied[11] and T has been shown to be metabolized to DHT in the prostate[5, 6, 7, 10]. The key intermediates belonging to C-19 steroids in the biosynthesis of T in testes have been shown to be dehydroepiandrosterone and 5-androstene-3β,17β-diol in the "5-ene" pathway and androstene-dione in the "4-ene" pathway. Each of these intermediates has been shown to be enzymatically interconvertible; the end product being T. 4-Androstene-3β,17β-diol has not been isolated in the testes so far; however, it is reasonable to assume that 4-androstene-3β,17β-diol is a more potent androgen *in vivo* than 5-androstene-3β,17β-diol because in the former only one enzymatic step is involved for its conversion to T (i.e. oxidation of 3β-hydroxyl to the keto group). Whereas in the latter it would involve two enzymatic steps for its conversion to T (i.e. oxidation of 3β-hydroxyl to the keto group and isomerization of the double bond to the more stable conjugated position). So the end organ response of these two isomeric diols seems to be due to their metabolism to an active metabolite (T or DHT). This hypothesis is supported by the data of Bruchovsky[10] who demonstrated the *in vivo* conversion of T, 5α-androstane-3α,17β-diol, androsterone and androsten-dione to DHT in the rat prostate cytosol. 5α-androstane-3α,17β-diol, androsterone and androsten-dione to DHT in the rat prostate cytosol.

In general, it has been observed by Liao[9] and confirmed by us that the presence of 17β-hydroxyl group in DHT is necessary for binding to the cytosol receptor proteins. These experimental data support the hypothesis proposed by Bush[19] who emphasized the importance of β-hydroxyl group at 17-position for receptor interaction and androgen response.

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