ANDROGEN BINDING AND ANDROGEN EFFECT IN THE RAT SEMINAL VESICLES: STUDIES WITH TESTOSTERONE AND 19-NORTESTOSTERONE

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SUMMARY

In an attempt to correlate androgen bdining and androgen effect, the seminal vesicle of the castrated rat was used as a model system. Competitive binding studies were carried out by incubating the mince of this gland with [³H]-testosterone and various concentrations of 19-nortestosterone (N) or testosterone (T). The binding of [³H]-dihydrotestosterone ([³H]-DHT) to cytosol proteins and nuclei was measured. To compare the competing capacity of N and T, the binding data were graphically analyzed, and the relative competition indices: N/T = 0.44 and 0.40, respectively, were obtained for cytosol binding and nuclear retention.

In order to estimate the androgen effect, the increase of the RNA/DNA ratio in response to the *in vivo* administration of the phenylpropionate esters of these steroids was determined. An analysis of the double reciprocal plot of data showed that the biological activity of N, relative to T, is 0.42, which is strictly consistent with the relative binding of these steroids (and/or their metabolites) in this tissue.

Control experiments have demonstrated that the rate of conversion of [³H]-T to [³H]-DHT is identical irrespective of whether N or T is present during incubation. It has also been found that N and T are released and absorbed from their phenylpropionate esters very similarly, although the rate of these processes is markedly influenced by the vehicle used.

INTRODUCTION

The widely accepted view of the mechanism of steroid hormone action involves the formation of a cytoplasmic receptor–steroid complex, the translocation of this complex to the cell nucleus and the initiation and/or acceleration of the synthesis of ribonucleic acids within the nucleus. Therefore, it may be assumed that following testosterone injection to castrated rats, the acceleration of RNA synthesis in their accessory sexual glands is exclusively dependent on the number of receptor–steroid complexes available for the primary sites of androgen action. Studies with antiandrogens [1] and with genetically androgen insensitive mice, rats and men [2] point to an essential role of a specific receptor in the mechanism of action of testosterone in male accessory sexual glands as well as in other androgen responsive tissues. Although previous studies suggest a correlation between receptor binding affinity and biological potency of various androgenic steroids [3–8], a strict correlation between these parameters has not yet been established. For example, 19-nortestosterone, which is much less androgenic than testosterone [9, 10], was found to be as effective as testosterone in a nuclear retention assay carried out with prostate mince [5] and even more efficient than 5α-dihydrotestosterone (the active metabolite of testosterone) in carefully controlled binding assays performed with prostate cytosol [3, 6, 7, 11]. Previous studies have shown that the seminal vesicle of the castrated rat may be used as a suitable model system to compare the androgenicity and binding activity of various androgenic steroids [12, 13]. Possible advantages of this model include the determination of binding at 37°C in a cellular system and the estimation of androgenicity on the basis of the increase of RNA/DNA ratio which is essentially the measure of the acceleration of RNA synthesis in this gland [13].

Employing this experimental model, we compared in the present study the biological potency and binding activity of testosterone and 19-nortestosterone. A preliminary report of this work has appeared [14].

MATERIALS AND METHODS

Steroids. [1, 2, 6, 7(n)-³H]-testosterone (87 Ci/mmol) was obtained from the Radiochemical Centre (Amersham, England). Testosterone, 19-nortestosterone and the phenylpropionate esters of these steroids were the products of Richter Pharmaceutical Works, Budapest, and 5α-dihydrotestosterone of Merck (Darmstadt, GFR).

Animals and their treatments. Adult male Sprague–Dawley rats of 220–280 g body weight were purchased from LATI, Godollo, Hungary, and castrated via the

scrotal route under ether anaesthesia. Animals used for the receptor binding and steroid-absorption studies were on the 5th-6th day following castration. To determine the effect of steroids on vesicular RNA/DNA ratio, various groups of animals were treated with the phenylpropionate esters of either testosterone or 19-nortestosterone for 4 days prior to their sacrifice on the 17th day after orchidectomy. Steroid esters, dissolved either in sesame oil or in a mixture of ethanol–ethyleneglycol (1:1, v/v) were injected daily i.p. in a volume of 0.2 ml. Animals in the control groups received the vehicle only and were killed together with the rats treated with steroid. There was no significant difference between the RNA/DNA ratios of castrates being on the 13th and on the 17th day following orchidectomy.

Receptor binding and nuclear retention assays. Details of these procedures have been described [12]. Briefly, freshly prepared mince of seminal vesicles (0.8–1.5 g) was incubated in 10 ml of Krebs-phosphate medium [15] with 10 nM [³H]-testosterone and 10–100 nM competitor steroids (19-nortestosterone or unlabeled testosterone) for 60 min at 37°C in oxygen atmosphere. Control mixtures without competitor were run simultaneously. After incubation the reaction mixtures were cooled in an ice bath, the mince was rinsed three times with cold physiological saline and homogenized with medium A (0.33 M sucrose, 50 mM Tris–HCl, pH 7.5, 3 mM CaCl₂).

Cytosol fraction was prepared by high speed centrifugation (230,000 g_{av}) of the 900 g_{av} supernatant of the homogenized seminal vesicles. The 900 g pellet was washed with medium A and suspended in 35 ml of medium B (2.4 M sucrose, 3 mM CaCl₂). This suspension was layered over 5 ml of medium B and centrifuged in the Spinco SW 27 rotor at 22,500 rev./min for 60 min. The pellet (purified nuclei) were washed twice, with 30 ml of medium C (5 mM MgCl₂, 0.1 mM EDTA, 1 mM 2-mercaptoethanol, 50 mM NaCl, 20 mM Tris–HCl, pH 7.5) each time and suspended in medium C containing 1 M NaCl. This suspension was allowed to stand overnight at +4°C. Finally the nuclei were disrupted by sonication to yield the homogenized *nuclear fraction.* Recovery of nuclei was in the range of 30–55% (as calculated on the basis of DNA contents of the homogenate and the homogenized nuclear fraction).

Bound and free radioactivity of the cytosol fraction was separated by equilibrium dialysis. This method has also been used for the study of the specific binding of various steroids to their receptors [16–19]. Aliquots of the retentate and dialysate and of the homogenized nuclear fraction were successively extracted with diethylether, chloroform, and dichloromethane. After evaporating the solvent, the residue was co-chromatographed with unlabeled 5α-dihydrotestosterone on Kieselgel HF₂₅₄ (Merck, Darmstadt, GFR) thin layer plates using chloroform–diethylether (85:15, v/v) solvent system. Carrier spots detected by a brief exposure to iodine vapor were scraped off into counting vials, the gel was extracted with 1 ml methanol, and, after addition of 10 ml of a scintillation solution (0.4% PPO, 0.005% POPOP in toluene), the radioactivity was determined with a Beckman LS 355 liquid scintillation spectrometer.

Radioactivity bound to cytosol and nuclear receptors (c.p.m./mg protein and c.p.m./mg DNA, respectively) were calculated as per cent of the control. Competition values for testosterone and 19-nortestosterone were measured at the same time using the same pool of vesicular mince and the average competition values obtained with four separate pools of the vesicles were used for competition analysis.

Competition analysis. The method described by Liao *et al.*[5] was applied. If function Y is defined so that in the binding assays:

$$Y = \frac{\text{bound c.p.m. in the presence of a competitor}}{\text{bound c.p.m. in the absence of a competitor}}$$

then

$$Y = \frac{([^3H]\text{-}T)}{([^3H]\text{-}T) + \alpha(c)} .$$

Where ([³H]-T) and (c) are, respectively, the concentrations of [³H]-testosterone and the competitor in the assay system and α is the competition index (*CI*) characteristic of a competing steroid. We determined α for testosterone (T) and 19-nortestosterone (N) separately by plotting $1/Y$ as a function of $\frac{(c)}{([^3H]\text{-}T)}$ ("relative competition plot"), when α can be obtained from the slope. The relative competition index (RCI) for 19-nortestosterone is defined then as:

$$RCI_N = \frac{\alpha_N}{\alpha_T} .$$

The RCI_N value was determined for receptor binding as well as for nuclear retention of 19-nortestosterone.

Determination of RNA, DNA and protein. RNA and DNA were separated similarly to the procedure of Munro and Fleck[20]. Tissue mince equivalent to the seminal vesicles of 4–6 uniformly treated rats was homogenized with ice-cold 0.4 N perchloric acid (PCA). Precipitated material was successively washed twice with cold 0.4 N PCA, once with 0.1 M Na-acetate in 85% ethanol, once with 96% ethanol, and twice with ether. The ether dried powder was digested in 0.3 N KOH for 18–20 h at 37°C. DNA was then selectively precipitated by acidification with PCA to pH 2 at 0°C, hydrolyzed in 0.4 N PCA at 90°C for 15 min and measured by the diphenylamine reaction [21] using salmon sperm DNA as standard. Ribonucleotides remained in the supernatant after removal of DNA and were determined on the basis of their ribose content as measured by the method of Mejbaum[21]. Protein was determined by the method of Lowry *et al.*[22], using horse serum albumin as the protein standard.

Determination of relative biological activity. The effect of these androgens *in vivo* on the vesicular RNA/DNA ratio of castrated rats was used as a measure of their biological activity (potency). The RNA/DNA ratios measured after steroid treatment were related to the control ratio, i.e. to the RNA/DNA ratio obtained with castrates not treated with steroid, and from the values dose–response curves were constructed. These hyperbolic curves can be made linear according to the function:

$$\frac{1}{R} = \frac{S}{D} + \frac{1}{R_m}.$$

Where R means the relative RNA/DNA ratio value (control value $= 1$) obtained with a steroid treatment at D mg/day dose level, whereas R_m symbolizes the possible maximum of the relative RNA/DNA ratio which can be attained with that steroid using the aforementioned schedule of administration. $1/R$ versus $1/D$ plot of the data fits a straight line the slope of which is denoted by S in the equation. The intercept of this line at the ordinate is $1/R_m$. This kind of representation of data obtained with testosterone and with another androgenic steroid (for example nortestosterone), respectively, is called the relative biological activity plot.

The relative biological activity (potency) of nortestosterone can be determined from its relative biological activity plot, provided that the intercepts coincide at the ordinate:

$$\frac{N}{T} = \frac{1/S_N}{1/S_T} = \frac{S_T}{S_N}.$$

Determination of testosterone and 19-nortestosterone levels in blood plasma. Absorption of testosterone and that of 19-nortestosterone into the blood plasma were studied separately, using different groups of castrated animals. A competitive protein binding assay was employed using third trimester human pregnancy serum. About 10 ml of blood was collected via heart puncture, into a syringe containing 50 μl of 0.5 M EDTANa$_2$. After centrifugation, the plasma was decanted and made alkaline by adding 0.1 vol. of 0.5 N NaOH. Triplicate aliquots (0.2 ml–0.5 ml for testosterone and 0.5 ml–1.0 ml for 19-nortestoterone) were extracted twice with 5 ml dichloromethane (freshly distilled over phosphorpentoxide). The combined extract was evaporated *in vacuo* and the residue dissolved in 0.5 ml buffer A (0.1 mM EDTA, 0.5 mM 2-mercaptoethanol, 20 mM Tris–HCl, pH 7.4). To this, 0.5 ml of binding protein (pregnancy serum diluted 25-fold with buffer A and containing 40 nCi/ml ([^{3}H]-testosterone) was added and the mixture was incubated first for 10 min at 40°C, then for 10 min at 0°C. Unbound steroids were removed with charcoal (0.3 ml of a slurry composed of 625 mg Norit A, 65 mg Dextran T-70 and 100 mg gelatine in 100 ml buffer A). After 20 min in an ice-bath, the tubes were centrifuged and 0.5 ml of the supernatant was trans-
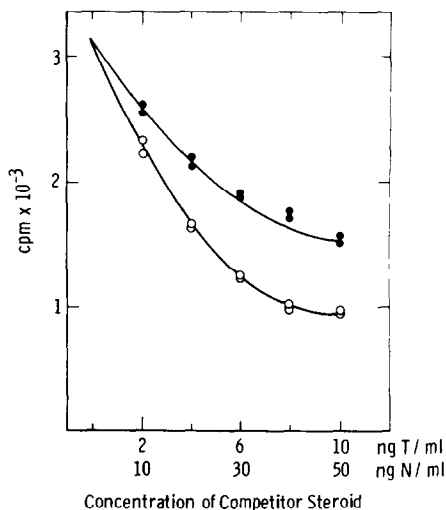

Fig. 1. Calibration curves for the determination of testosterone (O) and 19-nortestosterone (●) from blood plasma. Various amounts of steroids were dissolved in buffer A and 0.5 ml aliquots were used to determine competition using the standard procedure.

ferred to scintillation vials for the determination of radioactivity. Figure 1 shows typical competition curves obtained with standard solutions. Recoveries of testosterone and nortestosterone were 85–105%. Under the assay conditions the phenylpropionate esters of these steroids do not crossreact with the free steroid.

RESULTS

The competition of 19-nortestosterone and unlabeled testosterone with 10 nM [^{3}H]-testosterone for the binding to cytosolic and nuclear binding sites is shown in Fig. 2. Since more than 80% of the radioactivity bound to cytosol proteins and retained in the nuclei was recovered as dihydrotestosterone [12], we determined the label present in this steroid. On the other hand, we calculated the extent of the competition on the basis of testosterone and nortestosterone concentrations added to the incubation medium. This was necessary in order to compare the binding data with the *in vivo* effects of testosterone and 19-nortestosterone.

Binding data for these two steroids are presented in Fig. 2. From these relative competition plots were constructed (Fig. 3.). The relative slopes of best fit lines yielded RCI_N values of 0.44 and 0.40 for cytosol and nuclei, respectively. Data in Fig. 4 shows that the conversion rate of [^{3}H]-testosterone to [^{3}H]-dihydrotestosterone is identical irrespective of whether 19-nortestosterone or testosterone is present during incubation. Therefore, the observed differences in the competition values can be attributed to differences in the affinity of dihydrotestosterone and 19-nortestosterone (and/or its metabolites) to cytosolic and nuclear binding sites.
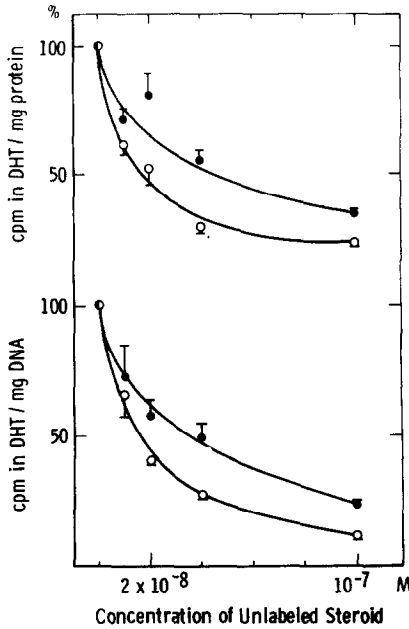
Fig. 2. Effect of various concentrations of testosterone (O) and 19-nortestosterone (●), respectively, on the binding of [³H]-dihydrotestosterone to cytosolic (top part) and nuclear (bottom part) binding sites. The vesicular mince was incubated for 60 min at 37°C with 10 nM [³H]-testosterone and competitor steroids as indicated on the abscissa. Each point represents the average of four determinations ± SD. Control values in the four sets of experiments were in the range of 1100–1500 c.p.m./mg protein (cytosol) and 12,000–16,000 c.p.m./mg DNA (nuclei). It has been shown that 6 nM testosterone in the incubation medium results in the saturation of these binding sites with dihydrotestosterone under these conditions [12].
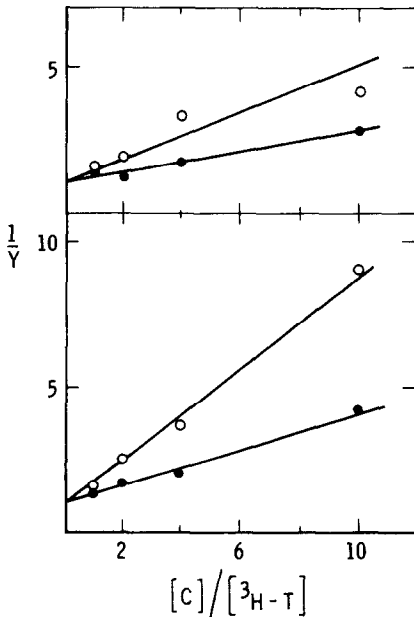

Fig. 3. Relative competition plot of data presented in Fig. 2 The competition index (CI) could be determined from the slopes separately for 19-nortestosterone (●) and testosterone (O). The relative competition indices (RCI) for 19-nortestosterone: 0.44 (cytosol) and 0.40 (nuclear retention) were calculated from CI values. Top: cytosol; bottom: nuclei.
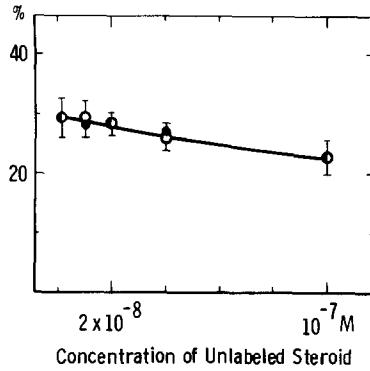

Fig. 4. Effect of various concentrations of testosterone (O) and 19-nortestosterone (●), respectively, on the conversion of [³H]-testosterone to [³H]-dihydrotestosterone. Aliquots (contining aboμt 3×10^4 c.p.m.) of the cytosol fractions obtained in the experiment described in Fig. 2 were extracted and analyzed to obtain the percentage recovery of radioactivity in the dihydrotestosterone fraction. Mean values ±SD are presented.

The relative biological responses of seminal vesicles to 19-nortestosterone and testosterone as measured by changes in the vesicular RNA/DNA ratio is illustrated in Fig. 5. These data provide evidence that the magnitude to which this tissue responds to androgens is markedly influenced by the nature of the vehicle
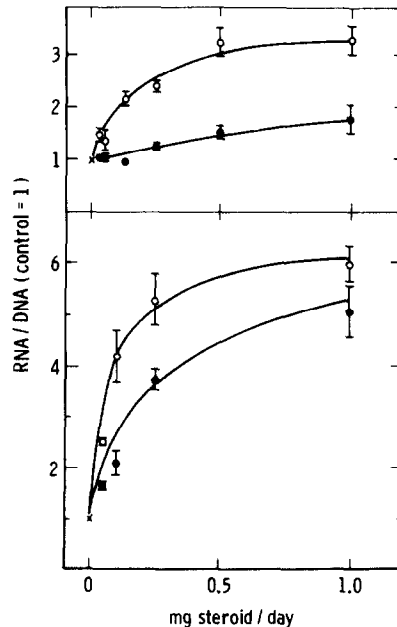

Fig. 5. Effects of treatment with phenylpropionate esters of testosterone (O) and 19-nortestosterone (●) on the vesicular RNA/DNA ratio of castrated rats. Dose-response curves obtained either with ethanol–ethyleneglycol 1:1 (v/v) (above) or with sesame oil (bottom) as solvent of the steroids are shown. Various groups of castrated rats (4–7 animals each) were given daily 0.2 ml solution i.p. for 4 days before killing on the 17th day following orchidectomy. The RNA/DNA ratios obtained with animals that received the vehicle only were taken as one unit and the changes relative to this unit (0.31 ± 0.055) were calculated. The mean values obtained from experiments with at least 5 different groups of animals, ±SD, are presented.
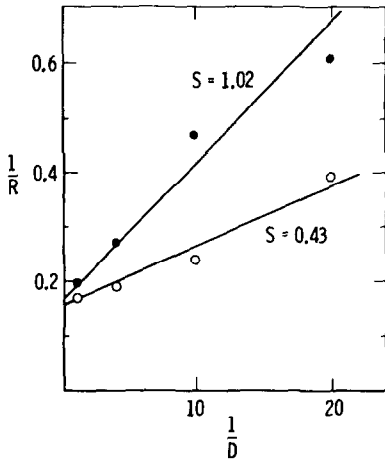
Fig. 6. Relative biological activity plot of data presented in the bottom part of Fig. 5. R = relative RNA/DNA ratios (RNA/DNA value of control castrates = 1), D = dose of steroid used for treatment.

used for injection. Applying the relative biological activity plot to the data of Fig. 5 is shown in Fig. 6 and Fig. 7.

It seems unlikely that the differences in the biological potency of these two steroids were due to differential absorption, transport, and/or metabolism, since plasma levels at different time intervals following injection were virtually identical (Fig. 8). However, the absorption and subsequent elimination of these steroids is apparently much faster when they are administered in ethanol-ethyleneglycol vehicle.

The relative biological activity can be estimated from the difference between the slopes and the intercepts. Further, from the intercepts, the maximal response (R_m) to steroid treatment can also be obtained. Thus, when sesame oil was used as solvent, an R_m value of 6.15 was obtained (Fig. 6) for both steroids (i.e., the intercepts at the ordinate coincided). Therefore, the ratio of slopes is the measure of relative biological potency of nortestosterone. This value (0.42)
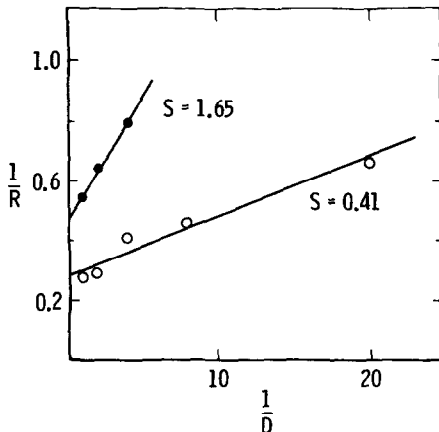

Fig. 7. Relative biological activity plot of data presented in the top part of Fig. 5. Symbols are the same as in Fig. 5 and Fig. 6.
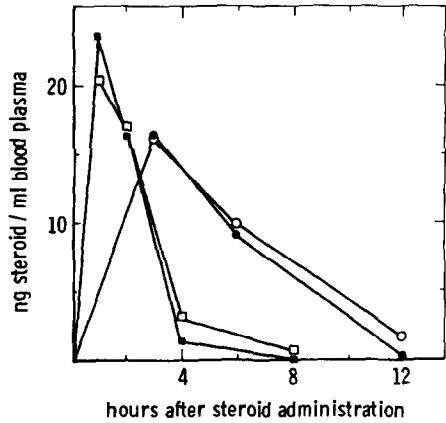

Fig. 8. Levels of testosterone and 19-nortestosterone in the blood plasma at various periods following intraperitoneal injection of their phenylpropionate esters. One mg testosterone phenylpropionate (open symbols) or nortestosterone phenylpropionate (filled symbols) was administered in 0.2 ml ethanol–ethyleneglycol (1:1, v/v) mixtures (squares) or in the same amount of sesame oil (circles). Single determinations with sesame oil as solvent were made at 1, 2 and 3 h; the range of these values was between 5.1–8.5 ng/ml. Each point is the average of 4–9 determinations.

suggests that testosterone is about 2.5 times more potent an androgen than 19-nortestosterone. This ratio is in good agreement with binding data (Fig. 3) suggesting a close connection between receptor binding and biological activity of these steroids.

In the case of ethanol-ethyleneglycol (Fig. 7), the ratio of slopes was $0.41/1.65 = 0.25$, indicating a 4-fold difference in relative potency in favor of testosterone. However, the R_m values were 3.57 and 2.13 for testosterone and nortestosterone, respectively, suggesting that during the latter treatment, the norsteroid did not saturate the system to the same extent as did testosterone. One can use this difference in the R_m values as a correction factor for the slope of the nortestosterone plot. The effect of nortestosterone is described by the following function:

$$\frac{1}{R} = \frac{1.65}{D} + \frac{1}{2.13}.$$

In order to correct the R_m value obtained for nortestosterone to the R_m value obtained for testosterone, the equation is divided by $\frac{3.57}{2.13} = 1.68$. In other words we substitute 3.57 (the R_m value for testosterone plot) for 2.13 (the R_m value for the nortestosterone plot) and we get the following equation:

$$\frac{1}{1.68R} = \frac{1.65}{1.68D} + \frac{1}{3.57}.$$

The corrected S value: $1.65/1.68 = 0.98$; and the corrected relative biological activity value: $0.41/0.98 = 0.42$ is again very close to the relative binding values obtained.

DISCUSSION

The experiments presented in this paper demonstrate a close correlation between the relative effect of testosterone and 19-nortestosterone on RNA synthesis and their relative ability to compete with [^{3}H]-testosterone to form cytosolic [^{3}H]-dihydrotestosterone-receptor complexes and to be retained in the nuclei of the rat seminal vesicle. As an extrapolation of this finding one may assume that the intensity of biological response is a direct function of the number of steroid-receptor complexes in the target cell. Applying the classification elaborated by Samuels and Tomkins[23] for steroids of glucocorticoid activity, the 19-nortestosterone apparently is an optimal inducer, since it or its metabolite(s) can saturate the dihydrotestosterone binding sites and can induce maximal response in RNA synthesis which is identical to that elicited by testosterone. To our knowledge, this is the first example in which a strict correlation between the binding and the biological potency of an androgenic analogue has been demonstrated. It should be noted, however, that Liao et al.[5] have examined a number of androgenic steroids and found a general tendency for such a correlation. It appears that binding studies carried out with cytosol preparations using reconstituted in vitro systems do not supply results comparable to the in vivo androgenic activity of a steroid. Both in the case of the prostatic [6, 7, 11] and the vesicular [24] cytosol, 19-nortestosterone was found to have an even higher affinity to the cytosol receptor than dihydrotestosterone. Other studies showed a lower affinity of this norsteroid to prostatic cytosol receptor when compared to that of dihydrotestosterone [4, 5], but the difference was relatively small, especially when compared to the remarkable difference found in the in vivo androgenic activity of these steroids.

It seems quite probable that 19-nortestosterone is metabolized in the male accessory sexual glands to a 5α-dihydro derivative, presumably by the same enzyme which reduces testosterone to 5α-dihydrotestosterone [25]. It has been shown for the prostate [5] and for the seminal vesicle [26] that the binding affinity of 5α-dihydro-19-nortestosterone to cytoplasmic receptor and nuclei is lower than that of 19-nortestosterone. Therefore, in cellular systems, such as the tissue mince used in our experiments, testosterone can be reduced to a derivative that is bound much more tightly to receptor sites while the 5α-reduction of 19-nortestosterone results in a steroid with diminished affinity for the receptor. (Expectedly, the equilibrium mixture of 19-nortestosterone and 5α-dihydro-19-nortestosterone which is present in the cells after some time of incubation is not so efficient a competitor as would be 19-nortestosteone itself in a reconstituted system.) It should also be borne in mind that the binding studies performed with cytosol have usually been made at or near to 0°C, and at 37°C the relative binding affinities of steroids may not be identical with those measured at lower temperatures.

The relative androgenicity value (NPP/TPP = 0.42) is comparable with the results of those experiments where the dose-response curves of vesicular weight gain in response to these steroid esters were established [27]. The measurement of the change of RNA/DNA ratio and the mode of evaluation of data as applied in this experiment seems to be a useful approach for the comparison of the androgenicity of various steroids.

It should be noted that the experiments presented here underline the importance of the "drug delivery system" [28] with respect to the intensity of the biological effect of androgenic steroids. However, it appears that by using the graphic analysis of the data, the responses can be interpreted unequivocally. The reason for the marked decrease in the response to steroids when ethanol-ethyleneglycol is used as solvent is unclear. However, the absorption profiles as well as the lower affinity of 19-nortestosterone (and/or its metabolites) for the receptors suggest that the retention of this steroid in the vesicular tissue might be less efficient than that of the dihydrostestosterone.

Recently, procedures have been reported for the measurement of occupied receptor sites in the prostate cytosol [29–31]. These procedures now make possible the search for a correlation between the number of occupied binding sites and the intensity of biological response following administration of testosterone to castrated rats.

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