

Androgenic effect of testosterone and some of its metabolites in relation to their biotransformation in the skin

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SUMMARY

Testosterone and several of its metabolites were tested in the female hamster flank organ. Microscopical examination was carried out and some histological features were quantified. Testosterone, 5α -dihydrotestosterone and 3α -androstane diol were very effective in stimulating the growth of the sebaceous structure, whereas 3β -androstane diol showed no effect at all. Androsterone could evoke only a small effect. The metabolism of these steroids was studied in the hamster flank organ, hamster skin and in human scalp hair follicles and the possible interrelationship between androgenic effect and metabolism is discussed.

The sebaceous glands of the hamster flank organ are stimulated by androgens. It has been suggested that 5α -dihydrotestosterone (DHT), rather than testosterone, is the active androgenic hormone in sebaceous glands (Takayasu & Adachi, 1972). DHT is formed from testosterone by the enzyme 5α -reductase. Local inhibition of the formation of DHT might be an effective means of blocking androgen action in the hamster sebaceous gland if indeed DHT is the only active androgen. However, some authors have suggested that androgens other than DHT are implicated in sebaceous gland activity. Ebling *et al.* (1973) indicated that in the rat there might be at least two distinct sites of androgenic control, mitosis and sebum synthesis; it was suggested that cell division might be controlled by a number of steroids, whereas 5α -androstane- 3β , 17β -diol regulated the secretion processes.

We have recently demonstrated (Vermorken, Goos & Wirtz, 1981) that the size of the pigmented spot and the sebaceous gland tissue show a differential response to topical androgen

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stimulation: the simultaneous application of testosterone and progesterone (in a dose sufficient to block the conversion of testosterone into DHT *in vitro*) resulted in an almost complete inhibition of the stimulatory effect of testosterone on the size of the pigmented spot, whereas no significant reduction of the sebaceous structure could be observed as compared to application of testosterone alone. This indicates that testosterone (or some other metabolite) may show an androgenic effect *per se*. Alternatively, it could mean that the applied dose of progesterone is insufficient to block the formation of DHT in deeper layers of the skin.

In this paper we describe the effect of testosterone and several of its metabolites on the hamster flank organ. In addition, the metabolism of these steroids both in the hamster flank organ, in adjacent hamster skin and in human hair follicles has been investigated. The possible interrelationship between the androgenic effect of these steroids and their metabolism is mentioned, and implications for the human situation are discussed.

METHODS

Steroids

[1 α ,2 α (n)-³H] Testosterone (specific activity 51 Ci/mmol), 5 α -[1 α ,2 α (n)-³H] androstane-3 β ,17 β -diol (specific activity 40 Ci/mmol), 5 α -[1 α ,2 α (n)-³H] androstane-3 α ,17 β -diol (specific activity 40 Ci/mmol) and 5 α -dihydro- [1,2,4,5,6,7,³H] testosterone (specific activity 101 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, England. [1,2-³H(N)] Androsterone (specific activity 40.8 Ci/mmol) was purchased from New England Nuclear. These steroids were checked chromatographically for radiochemical purity before each experiment. Unlabelled steroids were purchased from Sigma Chemical Company (St Louis, Miss., U.S.A.) and from Steraloids Inc. (Wilton, N.H., U.S.A.).

Hamster flank organ test

The hamster flank organ test was carried out as described earlier (Vermorcken, Goos & Roelofs, 1980a). The female hamsters were separated into eight groups of five animals each and treated according to the following scheme: group I, acetone (control group); group II, testosterone (T) (4 μ g); group III, T (40 μ g); group IV, 5 α -dihydrotestosterone (DHT) (4 μ g); group V, DHT (40 μ g); group VI, androsterone (ARON) (40 μ g); group VII, 5 α -androstane-3 α , 17 β -diol (3 α -diol) (40 μ g); group VIII, 5 α -androstane-3 β , 17 β -diol (3 β -diol) (40 μ g). As an extra control a group of five male hamsters (group IX) treated with acetone was used. After 23 days of treatment the flank organs were excised for morphometrical and histochemical examination.

Histology

For histological and morphometrical evaluations squares of shaven skin containing the flank organ were pinned in a flat position on cork plates, quickly frozen in liquid nitrogen (-190°C) and stored in a freezer at -90°C until further use. Cranio-caudal cross-sections (eight slides with each three sections of about 10 μm thickness per organ) were cut through the centre of the organ with a Walter-Dittes cryostat at -25°C . The sections were stored at -90°C for a maximum of 2 weeks. Sections were stained routinely with Paragon[®] (multiple stain for frozen sections) and with haematoxylin-eosin.

For morphometrical analysis a microscopic image of the organ was projected on a sheet of paper, at a fixed magnification, and drawn. The length of the organ was measured: the borderlines of the organ were drawn just outside the outer, larger sebaceous gland (compared with the small sebaceous glands of the normal skin). The drawings of the sebaceous glands were cut out with a swivel knife (Ulano[®], Switzerland) and the paper was weighed. In each gland

structure three regions were observed and weighed separately: the basaloid cell layer (regeneration zone), the degeneration zone and the central, sebum containing zone. Hair structures were classified into large hairs and small hairs, and counted in the largest cross-section.

Steroid metabolism

The metabolism of several steroids was investigated in human scalp hair follicles, hamster flank organ and hamster skin (females).

Human scalp hair follicles were plucked from several areas of the scalp and only those with visible bulb and sheath were employed. For each measurement, fifteen hair follicles were used. Hamster flank organs and pieces of hamster skin (of approximately the same size as the hamster flank organ) were excised and minced with the aid of scissors.

The samples were incubated with the various steroids in 0.2 ml 50 mM Tris-HCl buffer pH 7.5, containing 0.2 M sucrose, 100 units of penicillin G/ml and a NADPH-generating system (1 mM NADP, 20 mM glucose-6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase per ml and 0.5 mM $MgCl_2$). Radioactivity ($\pm 0.85 \mu Ci$) was added to the incubation mixture in 2 μl ethanol; the radioactive DHT was diluted with unlabelled DHT to a specific activity comparable with that of the other steroids. The samples were incubated at 37°C for 3 h under air.

The isolation and quantification procedure was as described earlier (Vermorcken, Goos & Roelofs, 1980b), with only a slight modification: for thin layer chromatography the plates were run twice in a solvent system of dichloromethane/ether (9:1).

RESULTS

The results of the application of testosterone and several of its metabolites to the female hamster flank organ are summarized in Table 1 and Fig. 1. On the basis of these data several remarks can be made:

(1) Application of testosterone or DHT results in a marked increase in the size of the pigmented spot (Fig. 1). The application of 4 μg of these steroids stimulates the growth of the pigmented spot to a value comparable with the value found with untreated male hamsters. A higher dose of 40 μg does not result in a further growth of the pigmented spot. From the results of the microscopical examination it can be seen that application of 4 μg of testosterone or DHT gives rise to an increase of the diameter of the sebaceous gland (to a value comparable with that of a male hamster) and the surface area of the sebaceous structure. However, this dose is insufficient to stimulate the latter parameter to the value of a male hamster. Application of 40 μg of testosterone or DHT results in a further increase of the surface area of the sebaceous gland (to the value found in untreated male hamsters).

(2) Application of 40 μg of testosterone or DHT results in a significant increase of the pigmented spot and the sebaceous gland at the contralateral flank organ.

(3) Application of 40 μg of androsterone results in an increase of the pigmented spot and the sebaceous gland, but the effect is much smaller than in the case of testosterone and DHT. At this dose androsterone exerts only a local effect: the contralateral flank organ shows no differences from a control flank organ.

(4) Application of 40 μg of 5 α -androstane-3 α ,17 β -diol gives an increase in the size of the pigmented spot and the sebaceous gland, comparable with the increase found after application of 40 μg of testosterone or DHT.

TABLE 1. Histological assessment of the response of the flank organs to treatment

Group	Treatment	Length (mm)	Surface of sebaceous structure*				Number of hairs†		
			Total	I	II	III	Total	Large	Small
<i>Treated flank organ</i>									
I	Acetone	3.96	365	12	21	332	8.0	0.5	7.5
II	4 µg T	5.60	1549	163	416	970	15.0	6.3	8.7
III	40 µg T	6.53	2717	202	1004	1511	26.7	9.0	17.7
IV	4 µg DHT	6.43	1996	226	630	1140	39.7	11.0	28.7
V	40 µg DHT	7.96	3460	362	1127	1971	21.6	5.3	16.3
VI	40 µg ARON	4.54	1181	100	310	771	19.6	3.3	16.3
VII	40 µg 3α-DIOL	7.59	2918	395	900	1623	30.7	6.7	24.0
VIII	40 µg 3β-DIOL	3.89	488	52	20	416	8.7	—	8.7
IX	Acetone (males)	5.42	3375	388	1246	1741	26.0	13.2	12.8
<i>Control flank organ</i>									
II	4 µg T	3.15	401	35	6	360	6.3	0.3	6.0
III	40 µg T	4.95	942	102	175	665	14.6	2.3	12.3
IV	4 µg DHT	4.26	591	49	40	502	13.0	1.0	12.0
V	40 µg DHT	5.32	1266	143	332	791	17.6	2.3	15.3
VI	40 µg ARON	2.73	417	29	12	376	9.3	—	9.3
VII	40 µg 3α-DIOL	4.72	1033	100	210	722	18.7	0.7	18.0
VIII	40 µg 3β-DIOL	3.61	391	37	8	346	13.0	—	13.0

* I = Sebum containing zone; II = degeneration zone; III = outer basal cell layer.

† Number of hairs counted in the largest cross section.

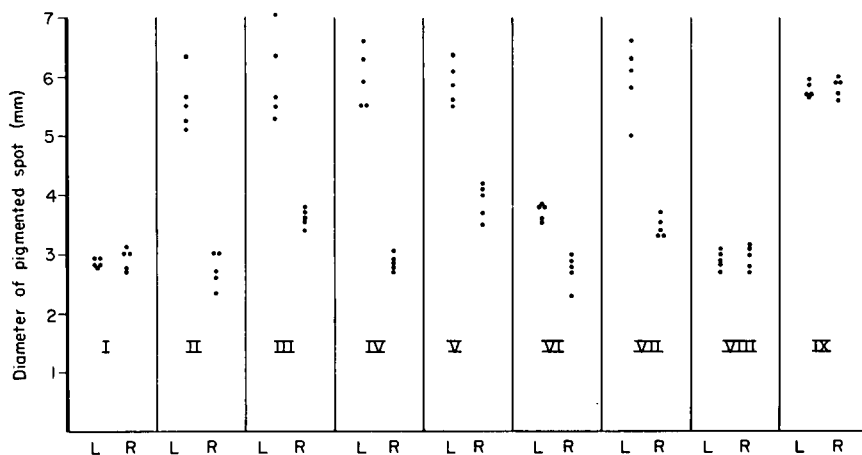


FIGURE 1. Visual assessment of the size of the pigmented spot of the left (L, treated) and right (R, control) flank organs of female hamsters. Groups I-IX are treated as described in 'Methods'.

TABLE 2. Metabolites of testosterone, incubated with human scalp hair follicles, hamster flank organ and hamster skin

Compound isolated	Human scalp hair follicles	Hamster flank organ	Hamster skin
5 α -DION	30.5 (1.28)	43.7 (1.11)	68.7 (1.61)
ADION	169.7 (7.12)	84.5 (2.14)	118.1 (2.78)
DHT	22.7 (0.95)	44.1 (1.12)	65.4 (1.54)
ARON (+ epi-ARON)	28.7 (1.20)	236.2 (6.01)	182.8 (4.30)
DIOL's	12.6 (0.52)	182.5 (4.63)	101.9 (2.40)
X	0.9 (0.04)	153.1 (3.89)	61.9 (1.45)
Origin	15.1 (0.63)	48.7 (1.23)	22.8 (0.54)

Quantities of radiometabolites are expressed as pmol formed/mg DNA.

Numbers in brackets represent percentage of substrate converted/incubation.

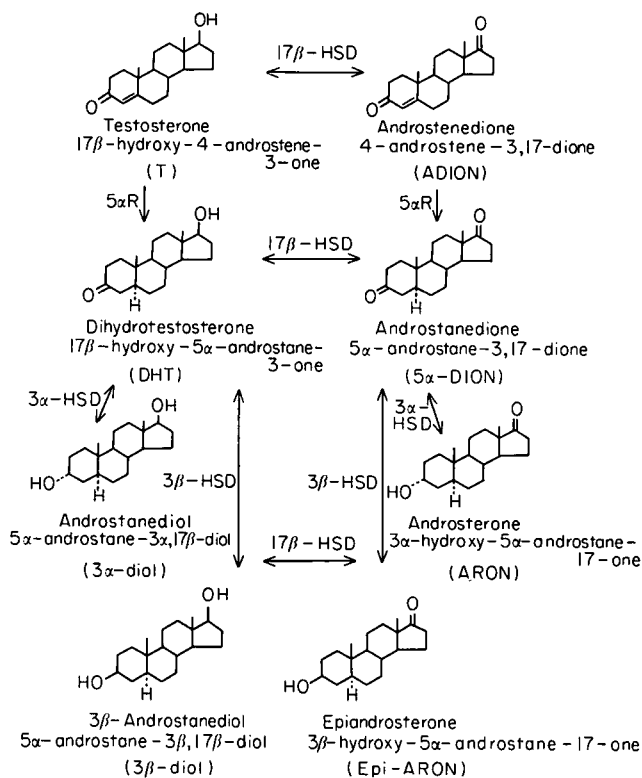


FIGURE 2. Metabolism of testosterone in the human skin. Abbreviations used for the enzyme reactions are: 5 α -R (5 α -reductase); 17 β -HSD (17 β -hydroxysteroid dehydrogenase); 3 α -HSD (3 α -hydroxysteroid dehydrogenase); 3 β -HSD (3 β -hydroxysteroid dehydrogenase).

(5) After application of 40 μg of 5α -androstane- $3\beta,17\beta$ -diol, no differences from a control group could be observed.

To investigate whether these effects could be attributed to the formation of a more active metabolite, the metabolism of these steroids was studied in hamster flank organ and adjacent hamster skin. As a control human scalp hair follicles were used. The metabolic products obtained on incubating testosterone with human scalp hair follicles, hamster flank organ and hamster skin are shown in Table 2. Figure 2 gives a schematic representation of the metabolism in human skin. All metabolites found in the radiochromatogram could be assigned except for a polar metabolite (designated X) in the hamster samples. In the TLC-system used 5α -androstane- $3\alpha,17\beta$ -diol and 5α -androstane- $3\beta,17\beta$ -diol could not be separated. Also, no separation could be demonstrated between androsterone and epiandrosterone.

The quantitative results of the incubation of the various samples with DHT are shown in Table 3. In Table 4 the results of the incubation experiments with androsterone as a substrate are summarized: in all samples virtually the same pattern of metabolites is formed. Only in the hamster samples, especially the hamster flank organ, is an extra, unidentified metabolite

TABLE 3. Metabolites of 5α -dihydrotestosterone, incubated with human scalp hair follicles, hamster flank organ and hamster skin

Compound isolated	Human scalp	Hamster flank	
	hair follicles	organ	Hamster skin
5α -DION	201.9 (9.50)	25.1 (0.72)	156.2 (4.09)
ARON	127.3 (5.98)	68.1 (1.86)	33.7 (0.88)
DIOL	268.0 (12.61)	2785.2 (77.88)	2115.5 (55.24)
Origin	36.8 (1.70)	49.1 (1.37)	39.1 (0.99)

Quantities of radiometabolites are expressed as pmol formed/mg DNA.

Numbers in brackets represent percentage of substrate converted/incubation.

TABLE 4. Metabolites of androsterone, incubated with human scalp hair follicles, hamster flank organ and hamster skin

Compound isolated	Human scalp	Hamster flank	Hamster skin
	hair follicles	organ	
5α -DION	34.7 (1.29)	399.8 (8.95)	411.3 (8.56)
DHT	6.1 (0.23)	15.7 (0.35)	12.0 (0.25)
DIOL	116.0 (4.31)	77.7 (1.74)	36.7 (0.77)
X	—	29.0 (0.65)	9.9 (0.21)
Origin	23.0 (0.86)	101.7 (2.28)	39.3 (0.82)

Quantities of radiometabolites are expressed as pmol formed/mg DNA.

Numbers in brackets represent percentage of substrate converted/incubation.

TABLE 5. Metabolites of 5α -androstane- 3α , 17β -diol, incubated with human scalp hair follicles, hamster flank organ and hamster skin

Compound isolated	Human scalp hair follicles	Hamster flank organ	Hamster skin
5α -DION	26.5 (0.98)	63.3 (1.42)	53.9 (1.12)
DHT	35.6 (1.32)	87.8 (1.97)	124.7 (2.61)
ARON	503.2 (18.66)	483.4 (10.91)	284.2 (5.94)
Origin	64.0 (2.38)	242.8 (5.46)	130.4 (2.72)

Quantities of radiometabolites are expressed as pmol formed/mg DNA.

Numbers in brackets represent percentage of substrate converted/incubation.

TABLE 6. Metabolites of 5α -androstane- 3β , 17β -diol, incubated with human scalp hair follicles, hamster flank organ and hamster skin

Compound isolated	Human scalp hair follicles	Hamster flank organ	Hamster skin
5α -DION	7.4 (0.27)	2.0 (0.05)	9.9 (0.21)
DHT	3.5 (0.13)	—	—
epi-ARON	71.5 (2.65)	1.7 (0.04)	7.3 (0.15)
Origin zone	637.2 (23.62)	4406.1 (98.58)	4182.6 (87.33)

Quantities of radiometabolites are expressed as pmol formed/mg DNA.

Numbers in brackets represent percentage of substrate converted/incubation.

(designated X) found. The results of the incubation of the androstane diols are shown in Table 5 (for 5α -androstane- 3α , 17β -diol) and Table 6 (for 5α -androstane- 3β , 17β -diol). A marked difference in the metabolic pattern of these two androstane diols could be observed: whereas the metabolism of the 3α -diol is practically the same for all samples tested and the metabolites of the 3β -diol correspond rather well with those of the 3α -diol, in the hamster tissue a very marked tendency towards the formation of more polar metabolites can be observed in the case of the 3β -diol.

DISCUSSION

The hamster flank organ test is widely used to measure the androgenic or antiandrogenic activity of topically applied compounds. In this test, as routinely used, the size of the pigmented spot is correlated with the effect of the compound applied. However, we have previously demonstrated that observation of the pigmented spot alone is insufficient for screening purposes. For better

evaluation of the androgenic or antiandrogenic effects of the compounds tested we introduced the quantitative measurement of several histological features (Vermorcken *et al.*, 1981).

From Fig. 1 it can be seen that topical application of 4 μ g of testosterone or DHT to female hamsters is sufficient to stimulate the growth of the pigmented spot to a value found in untreated males, whereas a much higher dose is necessary for maximal stimulation of the sebaceous glands (Table 1). It can be concluded that for stimulation of the sebaceous glands the dose-response relationship is maintained till a much higher dose, so that this measurement might be of more use for the testing of androgenic or antiandrogenic effects.

Very apparent is the difference in response of the application of the androstane diols, 3 α -androstane diol and 3 β -androstane diol. Whereas application of 3 α -androstane diol gave an effect comparable with testosterone and DHT, application of 3 β -androstane diol could not evoke any response at all. The metabolism of these steroids was studied to find out whether differences in metabolism might offer an explanation for this phenomenon. As a control the metabolism in human scalp hair follicles was also investigated. The metabolism of almost all steroids tested gave similar patterns of metabolites; all possible metabolites that are summarized in Fig. 2, including DHT, were formed. Only in the case of 3 β -androstane diol were differences found; a marked tendency towards the formation of more polar metabolites could be observed. The differences in metabolism of 3 β -androstane diol may explain the failure to cause any androgenic effect in the hamster flank organ. However, in human scalp hair follicles the metabolism of 3 β -androstane diol was clearly different; the metabolites correspond rather well with those of 3 α -androstane diol. This might indicate that the failure of 3 β -androstane diol to evoke any response in the hamster cannot be extrapolated to the human situation. Interspecies differences in metabolism may play an important role in the problems in extrapolating results from laboratory animals to the human situation; the human scalp hair follicle, an easily obtainable biopsy material, can be used to detect such differences before costly clinical tests are carried out.

The metabolism experiments show that DHT can be formed in the hamster flank organ from all steroids tested except from 3 β -androstane diol. Whether DHT is the only active androgen is doubtful; it has been suggested that numerous active metabolites are necessary for the full expression of the androgenic response. The failure of cyproterone acetate to inhibit all the processes mediated by testosterone and its metabolites is in agreement with this concept (Mainwaring, 1979). Metabolic inhibitors for the particular enzyme reactions that are involved in the metabolism of these steroids would be of great use to study the effect of steroids *per se*, but only few inhibitors are available. Until effective inhibitors are available, it remains difficult to assess whether the effects found are due to the steroid applied, or to possibly more active metabolites that can be formed in the target tissue.

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