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Observations on the Biological Activity of Epitestosterone

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Summary

Epitestosterone, a 17α-epimer of testosterone is a normal constituent of body fluids in many species including man. It has long been believed that it is devoid of any biological significance. However, it is now demonstrated that in *in vivo* experiments on castrated male mice it counteracts the action of testosterone on androgen-dependent organs. *In vitro* experiments show that on the overall antiandrogenicity of epitestosterone participate true antiandrogenic action due to the binding to androgen receptors, strong 5α-reductase inhibiting activity as well as a weak antigonadotropic activity. Epitestosterone is devoid of any embryotoxicity as checked by chick embryo-toxicity screening test.

Key words:
Epitestosterone – Antiandrogenic activity – 5α-reductase inhibition – Embryotoxicity – Receptor binding

Introduction

Epitestosterone (17α-hydroxy-4-androsten-3-one) was for the first time identified as an androgen metabolite in the rabbit liver as early as in 1947 by Clark and Kochakian. Later on it has been found to be a normal constituent of human plasma and urine and an endogenous C_{19}-steroid of many species. It is also a naturally occurring fytosterol, e.g. in the pollen of pine (*Pinus silvestris L.*). It was demonstrated that in humans, in males as well as in females, epitestosterone parallels approximately the levels of testosterone under various conditions, as age, sex, function of the gonads and hypo- or hyperandrogenic pathologic states. Though the production rate of epitestosterone is only 3 % of that of testosterone, its excretion rate is about 33 % of that of testosterone in the adult male (Wilson and Lipsett 1966). The nearly constant ratio of epitestosterone to testosterone of endogenous origin in the urine became the base of the control methods for testosterone doping, since epitestosterone does not originate from testosterone in significant amounts in the human and other species with low activity of 17α-hydroxysteroid dehydrogenase. Neither androstenedione interconversion to

epitestosterone and *vice versa* is of quantitative significance in species with poor activity of the above mentioned enzyme (Stárka and Breuer 1967, 1968). It is highly probable that the main portion of epitestosterone arises from testosterone precursors such as pregnenolone. A mechanism for 17α-hydroxy-C_{19}-steroid formation from pregnenolone was proposed by Weusten *et al.* (1989) as a satellite co-synthesis of 16-ene-C_{19}-steroids, the well-known pheromones and attractants of boars.

It has long been believed that epitestosterone is virtually devoid of any biological activity, especially no androgenic action could be demonstrated (Dorfman and Shipley 1956). There exists, however, a note indicating that epitestosterone is an inhibitor of 5α-reductase, an enzyme converting testosterone to an even more potent androgen for skin, kidney and accessory male sexual glands, dihydrotestosterone (Monsalve and Balquier 1977).

In 1987 Nuck and Lucky observed that epitestosterone when implanted subcutaneously, prevents androgenic stimulation of the flank organ of the female golden Syrian hamster as expressed by pigmentation, sebaceous gland growth and hair follicle diameter. As epitestosterone blocked not only the action of testosterone, presumably in part by 5α-reductase inhibition, but also in addition to it antagonized the action of dihydrotestosterone on flank organ growth, an effect that could not be accounted for by 5α-reductase inhibition, we tried to examine whether the epitestosterone action can be explained by another mechanism(s) besides 5α-reductase inhibition.

Methods

Bioassay for antiandrogenic activity

The test was based on antagonizing of the effect of 5 μmol testosterone propionate on the weight of seminal vesicles and on the total body weight of castrated male mice by a tenfold dose of epitestosterone or cyproterone acetate (a reference compound). The details are described elsewhere (Stárka *et al.* 1982).

In vitro binding to rat prostate androgen receptor

The relative binding affinity to androgen receptors was expressed as the ability of epitestosterone to displace [^{3}H]methyltrienolone from its specific binding to cytosolic receptor from prostates of castrated male rats, related to that of the above non-radioactive ligand, using log-logit plot. For details see Stárka *et al.* (1982).

In vitro inhibition of rat prostate 5α-reductase

The method consisted of incubation of [^{3}H] testosterone diluted with non-radioactive steroid to concentrations 0.40 – 3.2 μmol/l (4 points) with the pellet from homogenate of pooled rat prostates centrifuged at 800 x *g*, with or without epitestosterone (0.6 – 10.3 μmol/l), under optimal conditions for 5α-reductase activity (see Stárka *et al.* 1982, 1989). Following extraction with ethyl acetate, the metabolites were separated by thin layer chromatography (silica gel plates Alufol F_{254}, Merck, F.R.G., system cyclohexane–ethyl acetate 1:1) and the radioactivities, corresponding to the substrate and product (dihydrotestosterone) were measured by liquid scintillation counting.

The respective K_i values were calculated from Michaelis constants at increasing inhibitor concentrations as described by Brooks *et al.* (1981) and by Bartsch *et al.* (1987).

In vivo inhibition of gonadotropin secretion in the rat

The rats received vehiculum (controls), testosterone or epitestosterone (1 mg of steroid in 0.6 ml of olive oil each) in s.c. injections. On the morning of the first day after the last injection the animals were killed by ether narcosis and the blood was collected for determination of gonadotropins. LH and FSH were assessed in serum by radioimmunoassay, using human LH and FSH kits from the Institute of Radioecology and Application of Nuclear Technology, Košice, Czechoslovakia (LH) and SSW, G.D.R. (FSH). The cross reactions of the respective antisera with authentic rat LH and FSH (kindly donated by NIDDK under the Hormone and Pituitary Programme, University of Maryland, School of Medicine, Maryland, U.S.A.) were 3.6 and 4.1 %, respectively.

Chick embryotoxicity screening test (CHEST)

CHEST was carried out as described in detail previously (Jelínek 1977, Jelínek *et al.* 1985). Fertile eggs of the randombred White Leghorn population purchased from Velaz (Prague) were incubated in a forced-draught thermostatic oven at 38 °C and 40 – 60 % relative humidity. In the period before administration the eggs were rotated twice a day.

CHEST I (Estimation of the embryotoxicity dose range)

Following the first 40 h of incubation the eggs were candled, position of the embryo was marked on the egg shell and the eggs were opened using the common window technique. The technique consists in removing a rectangular piece of shell (cca 10 x 10 mm) within the area pre-sawn by means of a sharpened file for opening injection ampoules. After perforating the blunt end of the egg covering the air space, also the papyraceous membrane (membrana testae interna) was carefully removed from the window area. Developmental stage as well as the actual condition of the embryo were checked under a preparation microscope and externally normal embryos in developmental stages HH 10 – 11 were selected for the procedure. Test substances were injected into the subgerminal space in a physiological volume of 10 μl by means of a special glass calibrated microcannula with bevelled ground tip of 70 μm diameter. Subgerminal injections were chosen because in the above mentioned developmental stages no amniotic cavity, which serves as an obligatory injection route in embryos of the more advanced developmental stages (days 3 and 4), had already been formed. Each dose was administered to six embryos.

Following administration, the blastoderm was moistened by a single drop of chick saline (0.7 % NaCl), the window in the shell was covered with a glass slide on a paraffin frame, the window circumference was carefully sealed and the egg was returned into the oven and incubated for further 24 hours. During this period, eggs were slightly inclined from one side to the another twice a day avoiding any contact of the egg content with the cover-glass.

Twenty four hours after, eggs were taken out from the incubator and a distance between the anterior circumference of vitelline arteries and the tip of the tail was measured under a preparation microscope using an ocular micrometer at linear magnification 16x. At the same time, the degree of somitogenesis and closing of the anterior and posterior neuropores were checked as well. The distance measured corresponds to the length of the newly formed part of the trunk which has developed in the course of 24 hours in the presence of a given concentration of the test substance. The values were plotted into graph with logarithmic scale of doses (in ng) marked on the abscissa. In case of monotonous decline of a curve connecting median values or lengths of the trunk for particular doses, the differences were tested using nonparametric analysis of variance (Kruskal-Wallis test). Beginning of the embryotoxicity dose range is situated between the last ineffective and the effective doses.

CHEST II (Verification of the embryotoxicity dose range and construction of the embryotoxicity profile of the test substance)

The last ineffective and the initial two effective doses were administered subgerminally on day 2, and intraamniotically on days 3 and 4, to embryos in HH stages 11 – 14, 17 – 20, and 21 – 24, respectively. The procedure of egg opening was the same as in case of the estimation of the embryotoxicity dose range. At least 10 embryos for each dose and day of administration were used and their condition was checked on incubation day 8. Before checking, the embryos were carefully withdrawn from the incised extraembryonic membranes and weighed. The incidence of externally visible malformations of the head and face, the trunk, extremities, and, after microdissection even the heart and aortic-arch defects, were registered using a preparation microscope. Concerning the fact that the present experiment dealt with the testosterone analog, sex of each embryo surviving until day 8 was determined by inspecting the shape and dimensions of gonads. The results were summarized in a contingency table which, after calculating marginal totals and proportions, served as a basis for estimating the gross-response and stage-response relationships.

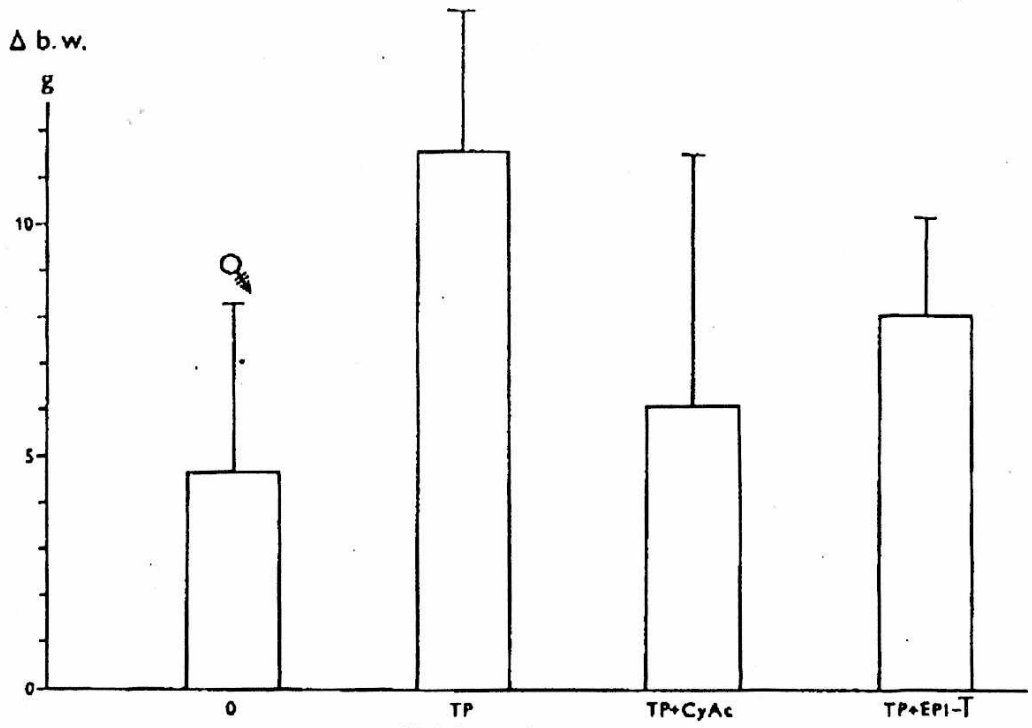

Fig. 1
Body weight increase of castrated male mice treated with testosterone propionate (TP) (5 μmol during 3 weeks) and epitestosterone (epi-T) or cyproterone acetate (CyAc) (50 μmol during 3 weeks). 0 = vehicle alone.

Results

Bioassay for antiandrogenic activity was carried out on castrated male mice to which testosterone propionate was administered in a dose approximately ten times lower than that of the antiandrogen. As reference compound cyproterone

acetate was used in a similar manner. The body weight increase and relative organ weights are shown in Fig. 1 and 2. Significance of the difference was calculated in relation to testosterone administration only and to the combination of testosterone and cyproterone acetate. Epitestosterone administration in a dose of 50 μmol during 3 weeks reduced the effect of testosterone propionate on body weight increment of castrated male mice. The relative organ weights (Fig. 2) indicated a significant antiandrogenic and antirenotropic activity. The relative weights of seminal vesicles and of kidneys were reduced in comparison to the group of castrated mice receiving testosterone propionate alone. The effect was even more pronounced than that of cyproterone acetate. Epitestosterone did not influence the relative weights of adrenals and spleen, in contrast to cyproterone acetate.

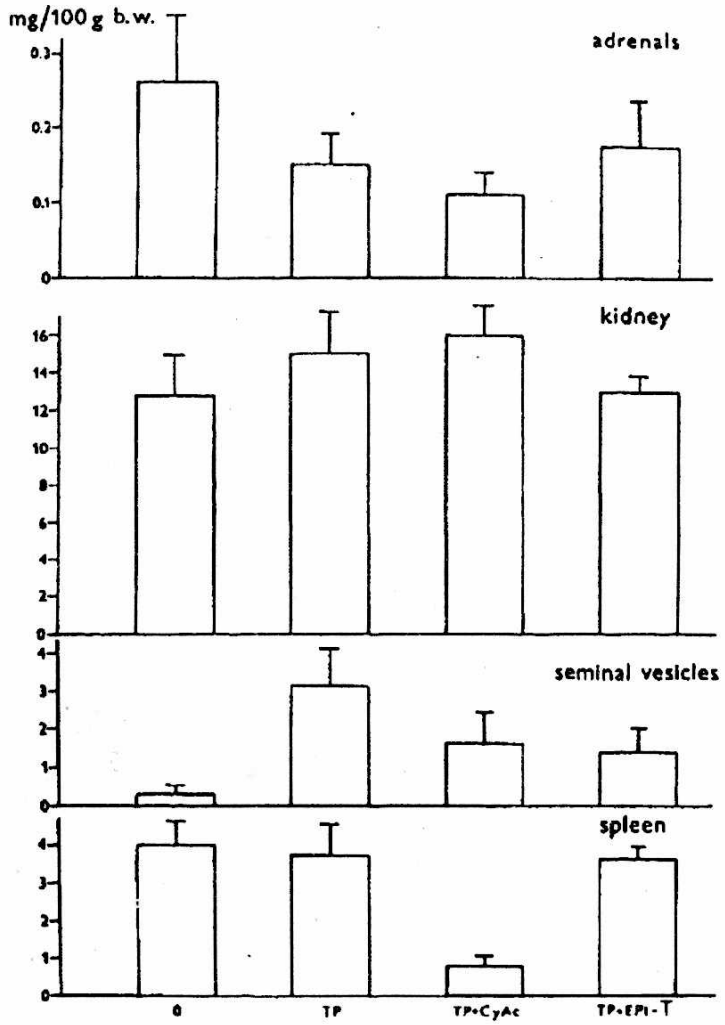

Fig. 2
Relative organ weights of adrenals, kidney, seminal vesicles and spleen of castrated male mice treated either with vehicle (olive oil) or testosterone propionate alone or in combination with epitestosterone (epi-T) or cyproterone acetate (CyAc) in doses as in Fig. 1.

The relative binding affinity of epitestosterone to rat prostate cytosol receptor using [^{3}H]methyltrienolone as a ligand amounted approximately to one third of that of the reference substance and one half of dihydrotestosterone (Tab. 1).

Table 1

In vitro competition of selected steroids with methyltrienolone for specific binding to rat prostate cytosol and the inhibition of 5α-reductase activity in rat prostate pellet

Steroid	K_i (nmol/l)	Binding to rat prostate cytosol % inhibition	Inhibition of 5α-reductase K_i (nmol/l)
methyltrienolone	9.0	100.0	–
epitestosterone	29.8	30.2	1.2
cyproterone acetate	22.0	40.1	no inhibition
progesterone	202.0	4.5	0.5
dihydrotestosterone	14.9	60.4	–

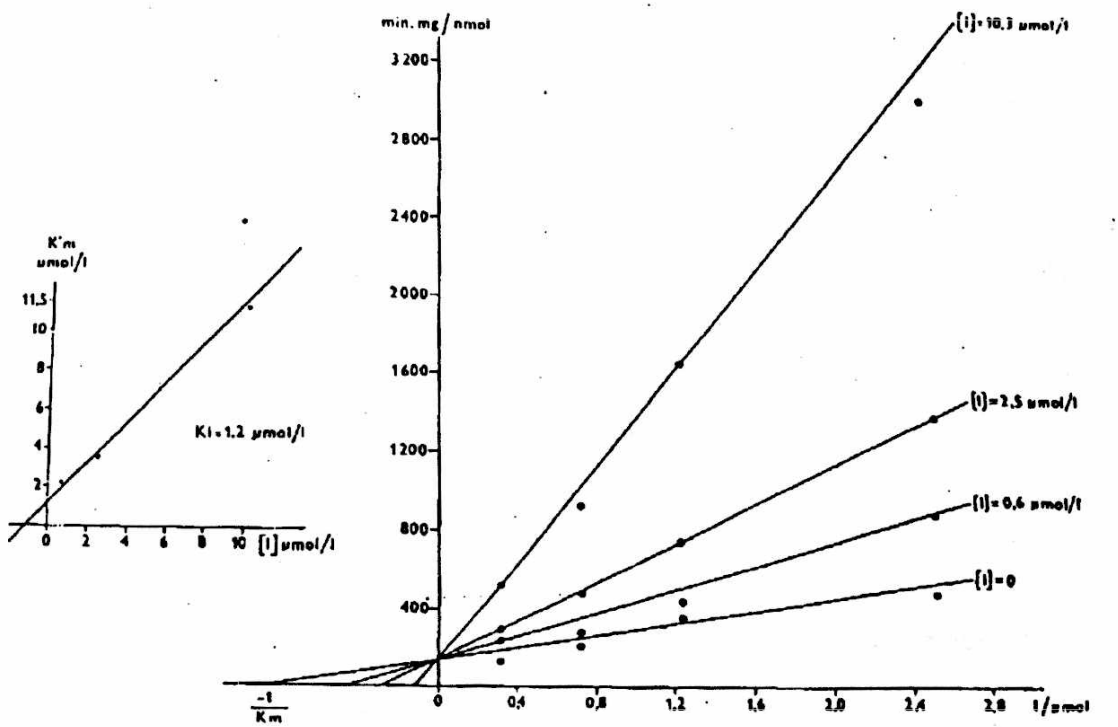

Fig. 3

Lineweaver-Burk plot of the inhibition of rat prostate 5α-reductase by epitestosterone (I). For experimental conditions see Experimental. Inhibitor was added at the concentrations indicated. Insert: plot of the apparent K_m. Values (K_m) vs the inhibitor concentrations (I), from which the inhibition constant K_i was read.

In vitro experiments with 5α-reductase from the rat prostate confirmed that epitestosterone is an effective inhibitor of the conversion of testosterone to dihydrotestosterone (Tab. 1). Epitestosterone behaved as a competitive inhibitor (Fig. 3) modifying the apparent K_m without alteration of V_{max}.

Epitestosterone administration to male mice decreased the secretion of FSH and LH (Fig. 4). However, the antigonadotropic activity of epitestosterone was only a moderate one.

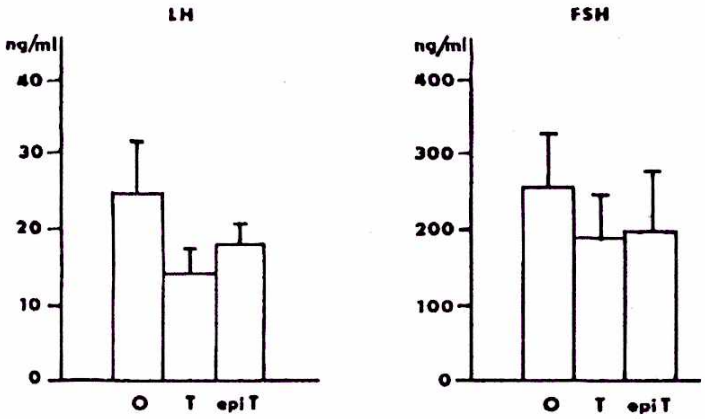

Fig. 4
The effect of epitestosterone *in vivo* on the secretion of LH and FSH in the plasma of male rats. Each animal received 1 mg epitestosterone for 4 days. Blood withdrawal was carried out next day following the last steroid administration.

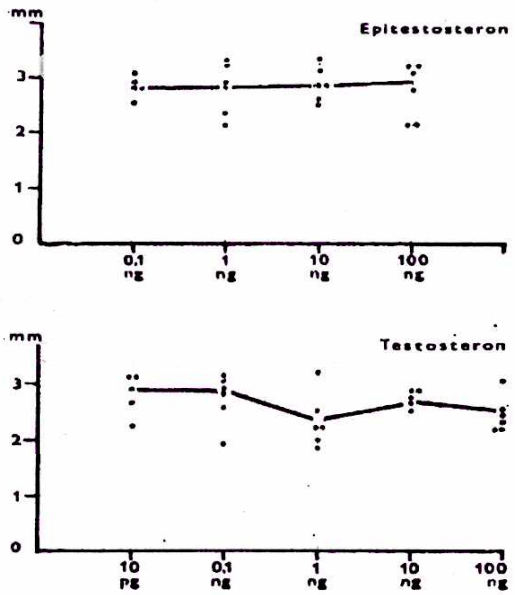

Fig. 5
Results of the embryotoxicity screening test CHEST I for epitestosterone and testosterone. Trunk length (in mm) vs. dose of steroid.

In the experiments for the estimation of the range of embryotoxicity there was no sign of a significant shortening of the newly developing part of the trunk of the embryos. It indicates that the function of the most active part of the embryo, i.e. of the caudal morphogenetic system, was not affected (Fig. 5). It means practically that the dose administered, as limited by the solubility of the steroids applied, did not reach the range of embryotoxicity.

Table 2
Results of embryotoxicity screening test CHEST II for epitestosterone

Dose/day	2nd day			3rd day			4th day			total			% of
	N	M	D	N	M	D	N	M	D	N	M	D	abnormalities
100 ng	9	1	0	9	1	0	10	0	0	28	2	0	6.7
10 ng	8	0	2	10	0	0	9	0	1	27	0	3	10.0
1 ng	9	0	1	10	0	0	10	0	0	29	0	1	3.3
total	26	1	3	29	1	0	29	0	1	84	2	4	6.7
%	87	3	10	97	3	0	97	0	3	93	2	4	6.7

Abbreviations used: N = normal, M = malformed, D = dead

Similarly as in the orientation test (CHEST I) even the complete assay for embryotoxicity as carried out with epitestosterone (CHEST II) did not show any embryotoxic effect in the range of the doses applied (Tab. 2 and 3). Epitestosterone did not influence the sex ratio, either.

Table 3
Foetal sex ratio and weight of embryos after epitestosterone and testosterone administration in higher dose on day 3

Compound	dose ng	number of embryos	results N	M	D	sex ratio male:female	weight of embryo ± S.D.
epitestosterone	520	47	46	1	0	24:23	1224 ± 78
testosterone	260	10	10	0	0	5:5	1244 ± 59

For abbreviations see Tab. 1

Discussion

Epitestosterone is a minor steroid metabolite and it has long been supposed to be biologically inactive. The observation that it could inhibit the 5α-reduction of testosterone to dihydrotestosterone appeared only as a marginal note in the literature (Monsalve and Balquier 1977). This activity was considered also as the main mechanism of the antiandrogenic activity of epitestosterone found in the experiments on the flank organ of the golden hamster by Nuck and Lucky (1987).

The present data show that the true antiandrogenic activity exhibited by the displacement of androgen from the binding to androgen receptor by epitestosterone is at least a part of the overall antiandrogenic action of this steroid. However, the inhibition of 5α-reductase remains an important factor of the effect. The inhibition was characterized more in detail as a competitive one (Stárka *et al.* 1989).

A further contribution of epitestosterone to the reduction of androgen supply of the organism is the antigonadotropic activity of the 17α-epimer of testosterone, shown in our experiments. Antigonadotropic activity of epitestosterone was postulated in a US patent as early as in 1959 (Allais and Hoffman).

From the practical point of view it is extremely important that epitestosterone did not exert any embryotoxicity. It is also worth further investigation as a drug for the treatment of hyperandrogenic states, especially in dermatology, e.g. acne, hirsutism or androgenic baldness.

Epitestosterone thus can be considered as an endogenous antiandrogen and as such to be associated to other endogenous steroids with antihormonal activity such as antimineralocorticoid progesterone, antiglucocorticoid 11-deoxycortisol or antiestrogen estriol.

References

ALLAIS A., HOFFMAN C.: U.S. Patent No.2,918,486, Dec. 22, 1959.

BARTSCH W., KLEIN H., STÜRENBERG H.J., VOIGT K.D.: Metabolism of androgens in human benign prostatic hyperplasia: aromatase and its inhibition. *J. Steroid Biochem.* **27**: 557–564, 1987.

BROOKS J.T., BAPTISTA E.M., BERMAN C., HAM E.A., HICHENS M., JOHNSTON D.B.R., PRIMKA R.L., RASMUSSON G.H., REYNOLDS G.F., SCHMITT S.M., ARTH G.E.: Response of rat ventral prostate to a new and novel 5α-reductase inhibitor. *Endocrinology* **109**: 830–836, 1981.

CLARK L.C., KOCHAKIAN CH.D.: The in vitro metabolism of testosterone to Δ^4-androstenedione-3,17, cis-testosterone and other steroids by rabbit liver slices. *J. biol. Chem.* **170**: 23–33, 1947.

DORFMAN R.I., SHIPLEY R.A.: Androgens. Wiley, New York 1956.

JELÍNEK R.: The chick embryotoxicity screening test (CHEST). In: *Methods in Prenatal Toxicology.* D. NEUBERT, H.J. MERKER, T.E. KWASIGROCH, (eds), G. Thieme Verlag, Stuttgart, 1977, pp. 381–386

JELÍNEK R., PETERKA M., RYCHTER Z.: Chick embryotoxicity screening test – 130 substances tested. *Indian J. Exp. Biol.* **23**: 588–595, 1985.

MONSALVE A., BALQUIER J.A.: Partial characterization of epididymal 5α-reductase in the rat. *Steroids* **30**: 41–51, 1977.

NUCK B.A., LUCKY A.W.: Epitestosterone: a potential new antiandrogen. *J. Invest. Dermatol.* **89**: 209–221, 1987.

STÁRKA L., BIČIKOVÁ M., HAMPL R.: Epitestosterone – an endogenous antiandrogen ? *J. Steroid Biochem.* **33**: 1019–1021, 1989.

STÁRKA L., BREUER H.: Vergleichende Untersuchungen über den Stoffwechsel von Epitestosteron im Testisgewebe der Ratte, des Kaninchens und des Stieres. *Z. Physiol. Chem.* **348:** 808–814, 1967.

STÁRKA L., BREUER H.: Vergleichende Untersuchungen über den Stoffwechsel von 17-Epitestosteron und Testosteron in Schnitten und Zellfraktionen der Rattenleber. *Z. Physiol. Chem.* **349:** 1698–1710, 1968.

STÁRKA L., HAMPL R., KASAL A., KOHOUT L.: Androgen receptor binding and antiandrogenic activity of some 4,5-secoandrostanes and ring-B cyclopropanoandrostanes. *J. steroid Biochem.* **17:** 331–334, 1982.

WEUSTEN J.J.A.M., LAGEMAAT G., VAN DER WOUW M.P.M.E., SMALS A.G.H., KLOPPENBORG P.W.C., BENRAAD T.J.: The mechanism of the synthesis of 16-androstenes in human testicular homogenates. *J. Steroid Biochem.* **32:** 689–694, 1989.

WILSON H., LIPSETT M.B.: Metabolism of epitestosterone in man. *J. Clin. Endocrinol. Metab.* **26:** 902–914, 1966.

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