Urological Research 2, 143-148 (1975)

A Systematic Study of Testosterone Metabolism in Benign Prostatic Hypertrophy (BPH): In Vitro Results*

J.E. Altwein and F. Orestano

Urologische Universitätsklinik, Mainz, Germany (FRG)

Received: April 23, 1974

Summary. An in vitro system for testing steroids which might be effective in treating benign prostatic hypertrophy (BPH) has been developed based upon the transformation of H^3 - testosterone into the 5α-reduction products dihydrotestosterone and 3α-androstanediol. In scrutinizing the influence of the amount of BPH-tissue, time, and pH, 300 mg of tissue incubated for 2 h at the physiological pH of 7.4 were used in the standard experiment. - The H^3 - testosterone concentration was varied from 0.17 - 100 x 10^8 M. Plotting the resulting 5α-reduction products as a function of testosterone concentration a hyperbolic pattern of enzyme kinetics ensued. Performing a double reciprocal plot of 4 experiments with double determination of each value regression lines could be computed. Those two regression lines most different in their slopes were considered "normal" limits. The rate of H^3 - testosterone metabolism could not be enhanced after the endogenous testosterone content within the prostate glands had been used up by means of a preincubation. Scrutinizing the effect of heparin, a weak non-specific enzyme inhibitor, no suppression of the appearance of 5α- reduction products was found. Damaging the BPH-cells, however, by repetitive freezing and thawing lead to an almost complete inhibition of H^3 - testosterone turnover.

Key words: Benign prostatic hypertrophy, testosterone, in vitro test system.

Introduction

The growth promoting and differentiating effect exerted by testosterone (T) is well established. The present knowledge has been gained as a result of investigations of the androgenic action upon BPH in the human prostate gland in vitro. Thus the 17 ß - hydroxy steroid dehydrogenase, 3 α - hydroxy steroid dehydrogenase, delta ^{4} - 3 - ketosteroid 5 α - reductase, 3 ß - hydroxy steroid dehydrogenase, and the delta ^{4} - 3 - ketosteroid 5 ß - reductase were discovered in BPH (2, 3, 14). Of these five enzymes the 5 α - reductase is of utmost importance in transforming T into 5 α - dihydrotestosterone (DHT) which is the essential mediator of T action (12).

Thus suppressing the rate of appearance of DHT would antagonize the effect of the "pre"-hormone T on the susceptible cells of the prostate (1, 4, 9). Of the available steroids with gestagenic or antiandrogenic properties which have been found effective in this regard, the most suitable for the treatment of BPH might be selected according to the results of an in vitro test.

Herein attempts are described to standardize the rate of T metabolism in BPH - tissue in vitro.

Material and Methods

Chemicals. 7 α-H^3-testosterone (Amersham, Buckinghamshire, England), supplied in a benzene solution had a specific activity of 5 Ci/mmole and the impurity did not exceed 1% as determined by paper chromatography in benzene : petrol (80-100° C) : methanol : water (250 : 250 : 350 : 150; v/v). 4 - C^{14}

* Supported by the Helmut Horten foundation

- testosterone (Amersham) supplied in benzene : ethanol (9 : 1; v/v) had a specific activity of 59 mCi/mmole and was not more than 1% impure as determined by paper chromatography (systems cf. above).

The following unlabelled steroids served as references and carriers (1 mg/ml chloroforme) : testosterone (androst-4-ene-17 ß ol-3one; Serva, Heidelberg); dihydrotestosterone (5 α-androstane-17 ß ol-3one; Merck, Darmstadt); 5 ß-dihydrotestosterone (5 ß-androstane-17 ß-ol, 3-one; Merck, Darmstadt); andostenedione (androst-4ene-3, 17-dione; Merck, Darmstadt); androstanediol (5 α-androstane-3 α 17-diol); 5 ß-androstanediol (5 ß-androstane-3 α , 17 ß-diol); 5 α-androstanedione (5 α-androstane-3, 17-dione) and 5 ß-androstane-3, 17-dione; Ikapharm, Ramt-Gan, Israel).

The organic solvents were of analytical grade.

Incubation. The tissue of 12 prostatic adenomata as confirmed by histology was placed in ice-cold normal saline immediately after transvesical removal, blotted and sliced into dices (0.5-1 mm). The standard incubation procedure has been described previously (Altwein et al., 1974). In addition to this basic experiment the achievement of a steady state condition was attempted by varying the time, pH, and the amount of tissue. The H^3-T concentration was increased from 0.17 to 100 x 10^{-8}M performing each study in duplicate.

In the preincubation studies the standard incubation was done initially without the tracer. Thereafter the medium was discarded, the tissue rinsed with Ringers-phosphate-buffer, and the medium replaced containing the tagged T, glucose (10^{-2}M), and NADPH (5 x 10^{-4}M) (Boehringer, Mannheim). In 2 experiments heparin was added (500 and 100 units/ml U.S.P.). In one additional experiment the cells were destroyed by repeated freezing to -20° C and thawing.

Extraction and Counting. The incubation was terminated by adding 2 volumes of chloroforme: methanol (2 : 1; v/v) to the incubation mixture. After evaporation 2 ml normal saline (4° C) and 5 000 dpm C^{14}-T serving as internal standard were added. The tissue was minced by means of the Ultra-Turrax (Ika-Werke, Staufen) and transferred together with 10 ml chloroforme : methanol (2 : 1; v/v) into centrifugation vials and shaken at 150 rpm on a bottle shaker (Bühler, Tübingen). The lipids were extracted and backwashed according to Folch et al. (1956).

The whole extract is taken to dryness, reconstituted with chloroforme and an aliquot is spotted on silica gel sheets F 254 (Merck, Darmstadt). The sheets are chromatographed along with 10 mcg cold reference steroids in saturated tanks: first ascent chloroforme: methanol, 97.5 : 2.5 (v/v) at room temperature; second ascent dichlormethane : ethylacetate, 80 : 20 (v/v) again at room temperature. The delta^4-steroids were visualized by their absorption of light with a wavelength of 254 nm (Desaga, Heidelberg). The areas of radioactivity were outlined by means of a thin layer chromatogram scanner (Berthold, Bad Wildungen). Finally the sheets were sprayed with acetic acid : sulphuric acid : anisaldehyde (100 : 2 : 1; by vol.) and the color was developed by heating at 120° C for 15 min. The steroids were identified by recrystallization to a constant specific activity (Tab. 1; cf. Altwein et al.).

The position of the various steroids was marked, each strip was cut into 10 parts, and transferred into counting vials together with 20 ml of a 0.4% diphenyloxazole, 10% methanol toluene-solution. The radioactivity was assessed in a Liquid Scintillation Counter (Packard, Frankfurt). The background cpm was substracted and dpm was calculated by correction for quenching (counting effiency approx. 35%), interference in the H^3-channel by C^{14} (approx. 23% spillover for the C^{14}-testosterone standard), and recovery of the C^{14}-internal standard (55 - 65%).

Results

As metabolic products of H^3 - T in BPH in vitro the subsequent androstanes were recovered and identified: T, DHT, 3 α , 5 α - and 3 α , 5 ß - androstanediol, androstenedione, 5 ß - dihydrotestosterone, 5 α -, and 5 ß - androstanedione. Identification of polar and non-polar C 19 - steroids was not attempted. Quantitatively T, the 5 -reduction products DHT, 3 α , 5 α -androstanediol, and androstenedione accounted for approximatively 85% of the radioactivity discovered (Fig. 1).

Furthermore, less than 1 pmole 5 α - reduction products or testosterone - androstenedione interconversion was observed when no tissue was added to the incubation medium (Fig. 1).

In looking for an appropriate amount of tissue as to the attainment of a steady state turnover of H^3 - T, 300 mg of BPH was found suitable (Fig. 2). In addition, it may be concluded from this experiment that H^3 - T enters the minute tissue dices by passive diffusion along a concentration gradient.

In Fig. 3 the rate of DHT plus 3 α , 5 α - androstanediol formation has been plotted as a function of time. After an initial steep increase in the formation of 5α -reduction products the curve levels off at 120 min. With regard to the pH-dependency, an acceleration of T turnover below the physiological pH of 7.4 is noticeable with a peak at pH 6.8 (Fig. 4).

In Fig. 5 the amount of 5 α -reduction products is plotted as a function of the T concentration. Each determination represents the mean of two values. The resulting graphs obey hyperbolic enzyme kinetics where at a substrate concentration of 100 x 10^{-8} M the marginal velocities (ordinate) range between 15 and 35 pmoles. Adding heparin, a non-specific enzyme inhibitor, the resulting velocities (quantity of 5 α -reduction products) slightly exceed the upper normal limit depicted in Fig. 5. A difference between 500 and 100 units/ml has not been measured (cf. below).

Table 1. Resulting cpm of recrystallization of C 19 - steroids recovered after incubation with human BPH - tissue

	System	Androstanediol	Testosterone	Dihydro-testosterone	Androstenedione
Crystals	Methanol : H_2O	./.	1979	1922	2010
	Acetone : H_2O	1810	1900	1910	1980
	Benzene : n-Hexane	1760	1874	1875	1810
	Ethylacetate : Cyclohexane	1488	1790	1710	1800
	Diethylether	1485	1770	1700	1780
Mother liquor	Methanol : H_2O	720	830	1020	390
	Acetone : H_2O	650	740	904	350
	Benzene : N-Hexane	640	630	910	340
	Ethylactate : Cyclohexane	555	690	880	295
	Diethylether	510	740	860	./.

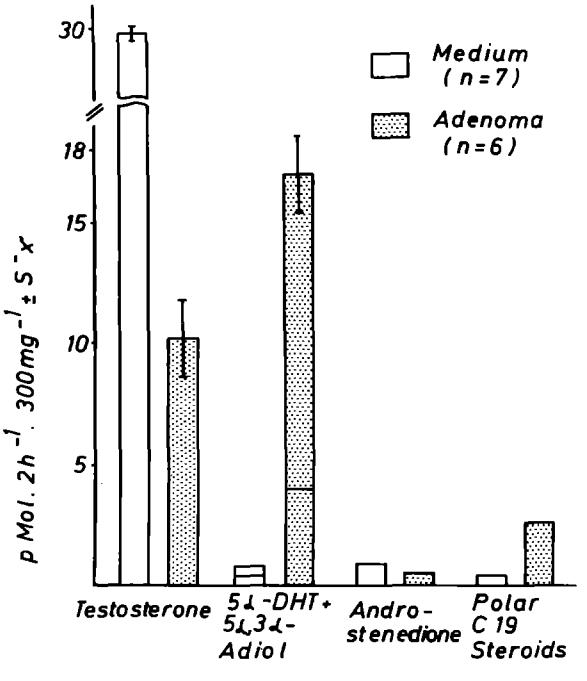

Fig. 1. Comparison of H^3 - testosterone degradation in BPH and medium alone. Initial testosterone concentration: 34 pmoles. On top of each column the SEM is indicated. 5 DHT-dihydro-testosterone; 5 α , 3 α - Adiol = 5 α - androstanediol

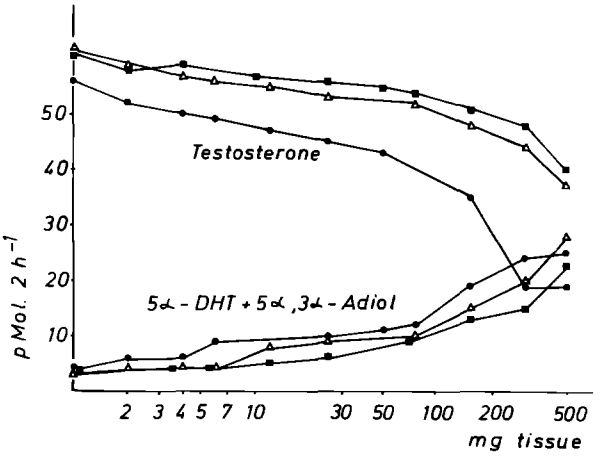

Fig. 2. Formation of 5 α -reduction products as a function of the amount of tissue (wet weight; semilogarithmic)

However, damaging the intraprostatic enzymes by exposing the tissue to repetitive freezing and thawing resulted in a significant decline of T metabolism (cf. below).

Preincubation without the tracer should utilize the endogenous T opening receptor- and enzyme binding sites. The second incubation in the presence of H^3 - T failed to enhance the T turnover rate (Fig. 6).

By doing a double reciprocal plot of 1/v (v = velocity = concentration of DHT and 3 α, 5 α-androstanediol/300 mg tissue/2 hrs) versus 1/ S (S = substrate concentration = H^3 - T added to the medium) of the data depicted in Fig. 5 the Fig. 7 is obtained. The shaded area represents the normal limits of T metabolism. Comparing these values with those obtained by performing a double reciprocal plot of the data obtained after incubation with heparin will result in the graphs of Fig. 8. There is obviously no difference in T metabolism with and without haparin in BPH. The dotted line (tagged PA 11 in Fig. 8) reflects the

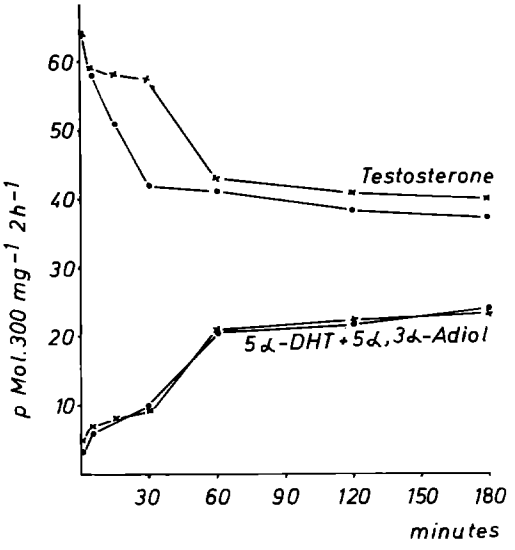

Fig. 3. Formation of 5 α-reduction products as function of time

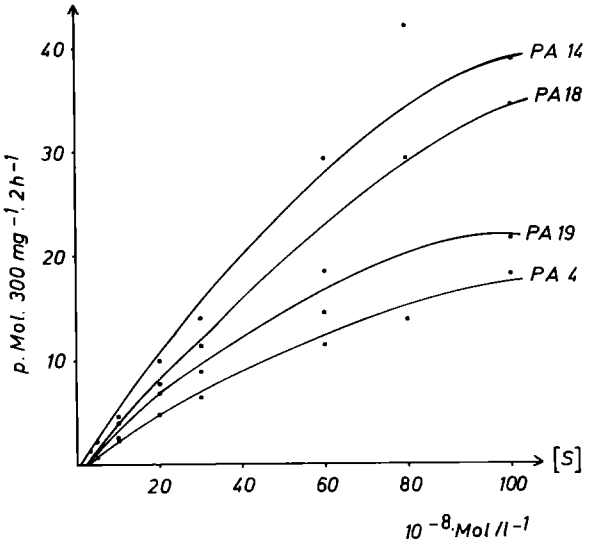

Fig. 5. Formation of 5 α-reduction products as function of H^3 - testosterone concentration. Each value represents the mean of two values. PA = prostatic adenoma = BPH. [S] = substrate concentration

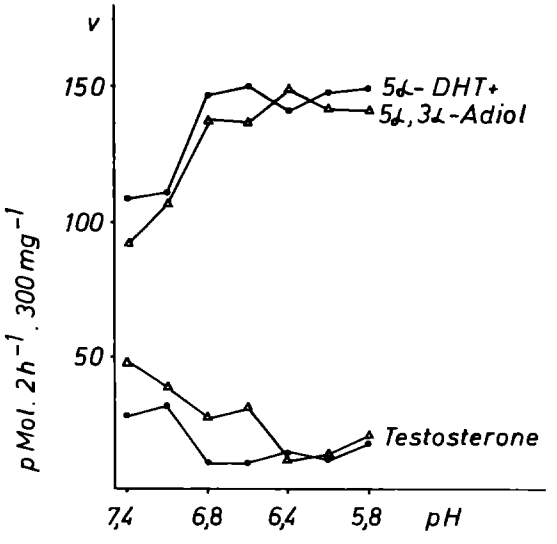

Fig. 4. Formation of 5 α-reduction products as a function of pH

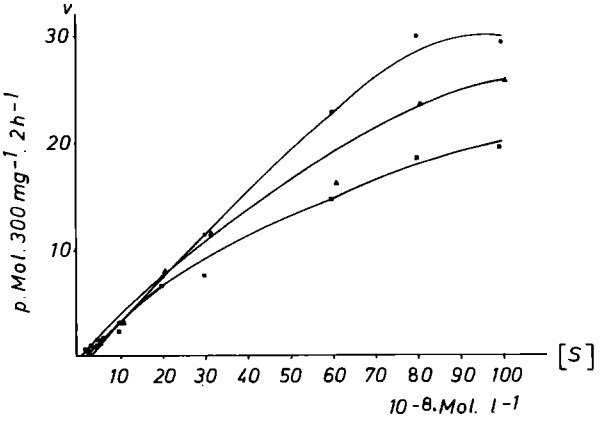

Fig. 6. The influence of preincubation upon H^3 - testosterone turnover. v = velocity.

enzyme inhibition due to alternate freezing and thawing.

Discussion

In this study the various factors influencing the in vitro metabolism of H^3 - testosterone in BPH were scrutinized. A standard model yielding sufficient reproducible results as the enzyme activity in non-homogenized tissue has been established. Thus the unfruitful attempts at homogenizing or even fractionating the BPH-tissue rich in collagenous fibres as hinted at by Mainwaring et al.(1973) is avoided.

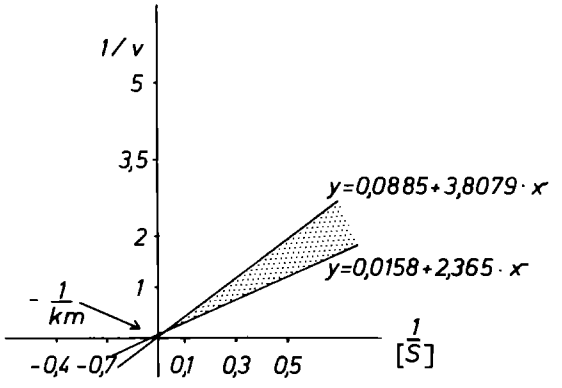

Fig. 7. Double reciprocal plot of the diagram in

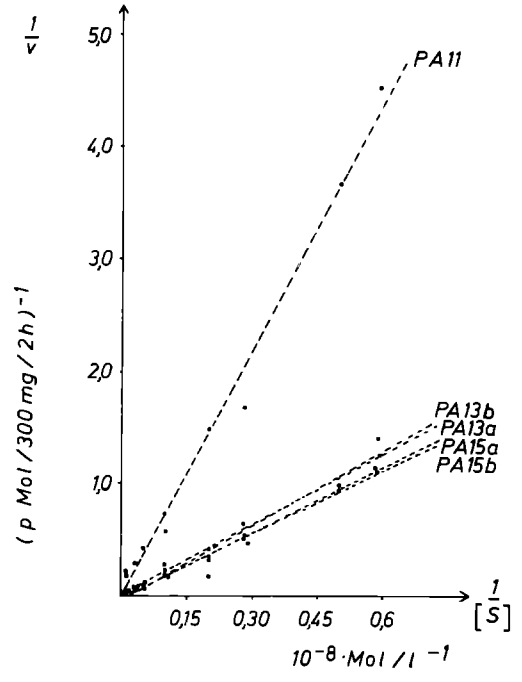

Fig. 8. Double reciprocal plot of the data presented in Fig. 6

Furthermore, there is no need to add cofactors which can influence the metabolic activity considerably (2). In using increasing amounts of tissue it has been demonstrated that the radioisotope diffuses evenly within the merged BPH tissue. Steady state conditions were attained as to time and pH in keeping with the findings of others (7, 13). The activity peak of the 5 α - reductase at pH 6.8 is in keeping with the observations made on the rat prostate (5).

The viability of the tissue appears to be maintained after short term incubation (11) making the use of organ cultures which have been used by Lasnitzki et al. (1974) for the ventral prostate of the rat unnecessary.

In incubations with increasing concentrations of H^3 - T a hyperbolic pattern of the formation of the 5 α - reduction products has been achieved comparable to the finding of Frederiksen and Wilson (1971) using rat ventral prostates. The rate of H^3 - T reduction was roughly linear between 5 and 60 x 10^{-8} M as has been described for the dog prostate homogenate (6). The double reciprocal plot of the reduction rate of H^3 - T may be used for the evaluation of the effect exerted by different drugs which appear to be of pharmacological interest. In a first approach heparin, a known non-specific enzyme inhibitor, was found to be relatively ineffective, whereas damaging the enzyme by freezing interfered considerably with the H^3 - T catabolism.

Using this system it has also been shown that "emptying" the endogenous T compartments within the BPH-cells did not augment the metabolic activity of exogenous T, i.e. H^3 - testosterone. This is apparantly due to the ratio of 600 : 1 between exogenous and endogenous T (11).

In essence, these experiments employing human BPH-tissue for scrutinizing the in vitro T- metabolism demonstrate the development of an vitro system for testing steroid pharmaca. In performing the double reciprocal plot for the formation of 5 α - reduction products of H^3 - T metabolism the degree and type of inhibition exerted by the steroid in question may be derived.

References

1. Altwein, J.E., Rubin, A., Klose, K., Knapstein, P., Orestano, F.: Kinetik der 5 α -Reduktase im Prostataadenom in Gegenwart von Oestradiol, Diäthylstilböstrol, Progesteron und Gestonoron - Capronat (Depostat). Urologe A 13, 41 (1974)
2. Chamberlain, J., Jagerinec, N., Ofner, P.: Catabolism of (4 - ^{14}C) testosterone by subcellular fractions of human prostate. Biochem. J. 99, 610 (1966)
3. Farnsworth, W.E., Brown, J.R.: Metabolism of testosterone by the human prostate. J. Amer. med. Ass. 183, 436 (1963)

4. Folch, J., Lees, M., Sloane Stanley, G.H.: A simple method for the isolation and purification of total lipides from animal tissues. J. biol. Chem. 226, 497 (1957)
5. Frederiksen, D.W., Wilson, J.D.: Partial characterization of the nuclear reduced nicotinamide adenine dinucleotide phosphate: Delta ^{4}-3-ketosteroid 5 α -oxidoreductase in the nuclear membrane of rat prostate. J. biol. Chem. 246, 2584 (1971)
6. Gloyna, R.E., Siiteri, P.K., Wilson, J.D.: Dihydrotestosterone in prostatic hypertrophy. II. The formation and content of dihydrotestosterone in the hypertrophic canine prostate and the effect of dihydrotestosterone on prostate growth in the dog. J. clin. Invest. 49, 1746 (1970)
7. Harper, M.E., Pike, A., Peeling, W.B., Griffiths, K.: Steroids of adrenal origin metabolized by human prostatic tissue both in vivo and in vitro. J. Endocr. 60, 117 (1974)
8. Lasnitzki, I., Franklin, H.R., Wilson, J.D.: The mechanism of androgen uptake and concentration by rat entral prostate in organ culture. J. Endocr. 60, 81 (1974)
9. Mainwaring, W.I.P., Milroy, E.J.G.: Characterization of the specific androgen receptors in the human prostate gland. J. Endocr. 57, 371 (1973)
10. Massa, R., Martini, L.: Interference with the 5 α -reductase system. Gynec. Invest. 2, 253 (1971/72)
11. Siiteri, P.K., Wilson, J.D.: Dihydrotestosterone in prostatic hypertrophy. I. The formation and content of dihydrotestosterone in the hypertrophic prostate of man. J. clin. Invest. 49, 1737 (1970)
12. Wilson, J.D.: Recent studies on the mechanism of action of testosterone. New Engl. J. Med. 287, 1284 (1972)
13. Wilson, J.D.: Testosterone uptake by the urogenital tract of the rabbit embryo. Endocrinology 92, 1192 (1973)
14. Wotiz, H.H., Lemon, H.M.: Studies in steroid metabolism. I. Metabolism of testosteron by human prostatic tissue slices. J. biol. Chem. 206, 525 (1954)

Dr. J.E. Altwein
Urolog. Univ.-Klinik
D-6500 Mainz
Langenbeckstr. 1
Federal Republic of Germany