METABOLISM OF 19-NORTESTOSTERONE BY HUMAN PROSTATE

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ABSTRACT

The metabolism of testosterone by human benign hypertrophic prostate to 19-norandrost-4-ene-3,17-dione (~1%), 19-nor-5α-androstane-17β-ol-3-one (8-15%) and 2-methoxyestrone (15%) is demonstrated. Incubation with a mixture of 1,2-^{3}H-testosterone and 4-^{14}C-19-nortestosterone was performed to determine to what, if any, extent 19-nortestosterone was an intermediate in the aromatization of testosterone by this tissue. From the yields and $^{3}H/^{14}C$ ratios of products it is tentatively concluded that, as in placental preparations, 19-nortestosterone and 19-norandrost-4-ene-3,17-dione play a very minor role in the aromatization process. It is noteworthy, also, that the saturated estrane, 19-nor-5α-androstane-17β-ol-3-one, appears to arise almost wholly from 10-demethylation of a saturated metabolite of testosterone, presumably, 5α-androstane-17β-ol-3-one. The significance of these findings, especially in relation to the Segaloff hypothesis is discussed.

INTRODUCTION

It has long been known that the administration of testosterone results in increased growth, biosynthetic and secretory activity in the prostate gland. In addition considerable evidence indicates that this tissue can metabolize testosterone and androstenedione to a number of other C-19 steroids (1, 2) and can also effect 10-demethylation of testosterone (3). As a consequence, one cannot say with certainty that testosterone, rather than one of its metabolites, is the biologically active androgen. This uncertainty confounds the efforts of those seeking to determine the mechanism

of androgen action, since tests of any steroid are conditioned by the ease with which this compound can (a) enter the cell, and (b) be metabolized to the biologically active form (assuming the tested compound is a precursor). Segaloff's recent finding (4) that a C-18 steroid derivative, 7α, 17α-dimethyl-19-nortestosterone, exhibited far greater androgenicity than testosterone in several bioassay systems, and Farnsworth's evidence of metabolism of testosterone to 19-nortestosterone (3), extends still further the number of compounds which may be the active compound.

This report is concerned with an investigation of the competence of the human prostate to metabolize 19-nortestosterone to other 19-norsteroids and to estrogens. The results of preliminary attempts to establish the metabolic pathways leading from testosterone to estrogen are also detailed.

MATERIALS

The 1,2-^{3}H-testosterone (New England Nuclear Corp.) and the 4-^{14}C-19-nortestosterone, (Nuclear Chicago) were partitioned between ether and 2N NaOH and the steroid in the neutral fraction subjected to gradient elution from alumina (see methods). Non-radiactive 19-nortestosterone was obtained from Preparations Labs, while 2-methoxy-estrone was purchased from Organon, Inc.

19-norandrost-4-ene-3,17-dione (I). This material was prepared from 19-nortestosterone by the method of Wilds and Nelson (5) (6) and recrystallized from cyclohexane to colorless plates, m.p. 167-70, $(\alpha)_D^{21}$ + 136.2 ± 2.3° (c = 4.9 in chloroform); λ_{max}^{KBr} 1739, 1672, 1621, 2924 (overtone) cm^{-1}, in accord with the literature (7). The dioxime of I was prepared by refluxing 50 mg. I 2 hours in .25 ml. pyridine with 50 mg. NH_2OH HCl in .25 ml. ethanol, diluting the cooled solution with an equal volume of water and evaporating off the solvent under nitrogen in vacuo. After preliminary recrystallization from dilute ethanol, the crude product was taken up in ethyl ether and repeatedly recrystallized with hexane, a modification of the procedure of Hartman et al (8), m.p. 145-7°; $(\alpha)_D^{21°}$ + 232.9 ± 25.7° (c = 0.12 in methanol); λ_{max}^{MeOH} 239 mμ, λ_{max}^{KBr} 3257 (OH), 1709, 1635 (CN) cm^{-1}. Anal.: Calcd. for $C_{18}H_{26}O_2N_2 \cdot 1/2\ H_2O$: C, 69.4; H, 8.7; O, 12.8; N, 8.9%. Found: C, 69.9; H, 8.4; O, 12.7; N, 10.0%. On thin layer chromatography on Silica Gel G with benzene-ethyl

acetate (4:1), the R_f of the dioxime was 0.193, of 19-norandrost-4-ene-3,17-dione, 0.33.

19-nortestosterone-17β-acetate. See (3).

19-norandrost-4-ene-3β, 17β-diol. Following the procedure of Bowers et al. (9), 19-nortestosterone (70 mg.) in dioxane (5 cc.) was treated with a solution of sodium borohydride (60 mg.) in water (0.2 cc.) and dioxane (1 cc.). After 2.5 hours at room temperature, the reaction mixture was diluted with 3 vols water, extracted (4X) with an equal volume of ether, the extract backwashed with water, dried with Na_2SO_4 and evaporated to dryness. The residue was taken up in hot acetone and recrystallized from dilute acetone, m.p. 169-70, $(\alpha)_D^{21^o}$ + 26.5 ± 2° (c = 3.7 in methanol), λ_{max}^{KBr} 3450(OH), 3400 (H-bonded OH), 1650 (C = C), 1053 (C-O of OH) cm^{-1}. Lit. (10).

19-nor-5α-androstane-17β-ol-3-one (II). This steroid was prepared from 19-nortestosterone (5 g.) by the procedure of Bowers et al. (9) m.p. 131-3°, $(\alpha)_D^{21^o}$ + 59.3 ± 1.3° (.23 in methanol), λ_{max}^{KBr} 3400, 3300 (OH) 1715 (C = O in 5-membered ring), 1075 (3-OH) cm^{-1}. Lit. (9): m.p. 130-131°, $(\alpha)_D^{21^o}$ + 60°. Acetylation of II (50 mg.) in pyridine (0.25 cc.) and acetic anhydride (0.5 cc.) overnight, followed by precipitation of the steroid in ice water and repeated recrystallization from dilute methanol and then from dilute acetone yielded crystals, m.p. 99-101°, $(\alpha)_D^{21^o}$ + 35.9 ± 1.2° (c. 4.7 in dioxane) λ_{max}^{KBr} 1715 (C = O), 3030 and 1255 (ester) cm^{-1}. Lit. (10): m.p. 102-4°; $(\alpha)_D$ + 36° (in dioxane); (7, No. 668) The R_f of this product, on thin layer chromatography with cyclohexane-ethyl acetate was 0.39; of the 5β epimer, 0.32.

19-nor-5α-androstane-3,17-dione (II). (100 mg.) was treated with CrO_3 (10 mg.) in glacial acetic acid (2.4 cc.) and water (0.1 cc.) overnight. The reaction mixture was diluted with iced distilled water (200 cc.) with stirring to precipitate the steroid. After drying the product by precipitation from dilute methanol, it was recrystallized from dilute acetone, m.p. 70-72°, $(\alpha)_D^{21^o}$ + 119.8 ± 3.3° (c = 0.6 in dioxane); λ_{max}^{KBr} 1745 (C = O in 5-membered ring), 1715 (C = O in 5-membered ring) 1453 (active methylene) cm^{-1}. Lit. (11): m.p. 70-3°; $(\alpha)_D^{21^o}$ + 122° (in dioxane); (7, No. 672).

19-nor-5α-androstane-3β, 17β-diol. This was prepared from II (100 mg.) by the method of Bowers et al. (9), omitting elution from alumina. It was recrystallized from dilute acetone, m.p. 168-172°, $(\alpha)_D^{19.5^o}$ + 24 ± 2.4° (c. 3.7 in MeOH). Upon solvolysis by repeated recrystallization from acetone, m.p. 169-73°, $(\alpha)_D^{20}$ + 14.9 ± 2.3 (c = .78 in $CHCl_3$), λ_{max}^{KBr} Lit. (9): m.p. 168-70°, solvated with two moles acetone, $(\alpha)_D$ + 10°.

2-Methoxyestrone-3-acetate (III). Acetylation of 2-methoxyestrone (50 mg.) in acetic anhydride (0.5 cc.) and pyridine (0.25 cc.) overnight, precipitation from 200 cc. iced distilled water and recrystallization from dilute acetone yielded crystals of the acetate (positive hydroxamate test) m.p. 153-4°, $(\alpha)_D^{22^o}$ +146.6 ± .2 (c = 12.8 in $CHCl_3$) λ_{max}^{MeOH} 279 mμ, infrared spectrum identical to the literature, R_f on Silica G with benzene-ethyl acetate (4:1), 0.75; R_f of 2-Methoxyestrone, 0.65. Lit. (12): m.p. 152-153.5 λ_{max}^{MeOH} 278 mμ (ε 2360); inflex, 281 mμ (ε2300); inflex, 287 mμ (ε2050); max 249 mμ (ε1040) in ethanol; I. r. in CS_2: bands at 1770 (phenolic acetate), 1743 (C-17

ketone), 1409 (unsubstituted methylene at C-16), 1375 (C-18 methyl group), 1370 (acetate methyl group), and 1198 (acetate C-O stretching) cm^{-1}.

2-Methoxyestradiol-17β-3-acetate. Fifty mg. III was reduced by the method of Bowers (9), and recovered from the reaction mixture by dilution with water and extracting into ether. On recrystallization from dilute methanol, m.p. 148-.5°, $(\alpha)_D^{20°}$ + 59.7 ± 13° (c = 0.24 in $CHCl_3$) λ_{max}^{MeOH} 281 mμ; I. r. KBr: Relative III, there is disappearance of the 1743 cm^{-1} band and appearance of a strong hydroxyl band at 3100 cm^{-1}. This product was saponified by treatment of 4 mg. in methanol (1 cc.) with 2.5% aqueous Na_2CO_3 (1 cc.) for 1 hour at room temperature. After evaporation of the methanol, 2-methoxyestradiol was extracted into $CHCl_3$ and, following evaporation of the solvent, recrystallized from acetone, m.p. 183-4°, $(\alpha)_D^{21°}$ + 91 ± 21° (c = 2.3 in chloroform), λ_{max}^{MeOH} 285 mμ, λ_{min}^{MeOH} 255. Lit. (13): m.p. 184-6 and 188-90°, $(\alpha)_D^{21°}$ + 100°, λ_{max} 280 mμ, λ_{min} 253 mμ.

METHODS

Human benign hypertrophic prostate, obtained by supra- or retropubic prostatectomy, was prepared and incubated as previously described (3). The pooled ether extracts of tissue and medium were washed three times with an equal volume of 2N NaOH and the alkali fraction backwashed with ether. After acidification with concentrated H_2SO_4, the alkali-soluble or phenolic steroids were partitioned into ether by three extractions, the ether washed with water, dried with Na_2SO_4 and evaporated at 40° in vacuo. The original, alkali-washed ether extract, which constituted the "neutral fraction," was similarly dried and evaporated.

Chromatographic systems: I, ligroin/ethylene glycol-methanol on 22-inch strips of Whatman 20 paper. II, o-dichlorobenzene-formamide. For further resolution of neutral steroids, the A and B-3 systems of Bush (14) were used to develop 45-inch strips of Whatman 2 paper in 4 foot chromatographic chambers for periods of 36 to 48 hours. Mahesh's System I (15), ligroin:benzene:methanol:water (67:33:80:20), and Axelrod's methylcyclohexane/propylene glycol system (16), "System III", were employed in further resolution of 2-methoxyestrone. The gradient elution column system was previously described (3) and the thin layer solvent systems used on Silica G are detailed in the results.

Detection and quantitation of steroids: Compounds containing 3- or 17-carbonyl groups were located on papergrams and assayed by reaction with an alcoholic solution of m-dinitrobenzene and KOH. The α,β-unsaturated steroids were detected by their absorbance in ultraviolet light and quantitated by measuring their optical density at 220, 240 and 260 mμ vs. appropriate reference standard compounds and applying the correction of Allen (17). Steroid alcohols were located on paper and thin layer chromatograms by exposing these to the vapors of iodine and were quantitated either by oxidation to ketones and use of the m-dinitrobenzene-KOH reagent or by acetylation and assay by the Baggett and Engel procedure (18). Phenolic steroids were detected on paper by the modified Millon reagent of Axelrod (19) or by the Turnbull blue reagent; on thin layer plates by absorbance under ultra-

violet light. They were quantitated by spectrophotometric measurement of absorbance at 260, 280 and 300 mμ and application of the Allen correction (17). When several mgs. of material were available, e.g. in recrystallizations, the crystals were dried to constant weight in vacuo and weighed on a Mettler Semi-Micro balance.

Paper chromatograms were scanned for distribution of radioactive material on an Atomic Accessories Scanogram I (Effiency without window: ^{3}H, 1%, ^{14}C, 5%). Strips containing doubly labelled products were rescanned with a window of Saran Wrap over the collimating slit. Calibration revealed that the presence of the film reduced the detector efficiency for ^{14}C by 1/3, for ^{3}H to 0. The effect on ^{14}C efficiency was linear; on ^{3}H, complete. As previously described (3), by counting a 10% aliquot of each extract before chromatography and estimation of the percent distribution of area above background in each radioactive peak from the integration tracing of the scanner, the activity of each product was determined. Applying this principle to quantitation of the doubly labelled products, the following calculations were employed:

^{14}C = 3/2 X apparent activity with window.

^{3}H = apparent activity in absence of window - ^{14}C activity.

The validity of these calculations was confirmed on chromatograms containing known quantities of a mixture of 1,2-^{3}H-testosterone and 4-^{14}C-testosterone.

Radioactivity in eluates of paper, column and thin layer chromatograms and in crystals was assayed in an Ansitron liquid scintillation spectometer employing external standardization and quench correction curves to convert cpm. to dpms. A PPO-POPOP solution in toluene was used for steroids, Bray's solution (20) for radioassay of tritiated H_2O.

RESULTS

Metabolism of 19-nortestosterone. (Experiment 1). In order to determine if the prostate is capable of accepting 19-nortestosterone as a substrate for further metabolism, one-gram lots of tissue slices were incubated 90 min. with 0.25 μc 4-^{14}C-19-nortestosterone (23.2 μc/μmole). Since 10-demethylated reference steroids were not then available, the alkali-washed ether extracts were mixed with authentic 19-nortestosterone, androst-4-ene-3,17-dione, 5α-androstane-3,17-dione and 5α-androstane-17β-ol-3-one and chromatographed in System I. The pattern of product distribution was both qualitatively and quantitatively similar to that obtained from incubations with testosterone. In general, each of the products of 19-nortestosterone was noticeably more polar than that product of testosterone.

Aromatization of testosterone (Experiment 2). To see if the products of 10-demethylation were limited to 4-unsaturated and saturated estranes or also included aromatic metabolites, tissue from five glands was incubated with 1,2-^{3}H-testosterone. The ether extracts were washed with 2N NaOH, and the alkali-soluble material was recovered by acidification and ether extraction of the aqueous phase. In addition, an aliquot of the incubation medium from four of the experiments was freeze-dried to determine the tritium level in the water. A portion of the water obtained by lyophilization of incubation medium from two of these experiments was fractionally distilled. The specific activity of water in all fractions was equal and no activity was found in washings of the distillation pot. Table 1 presents the details of these experiments and shows that from 10% to about 30% of the radioactivity appeared in the sum of the water and phenolic (alkali-soluble) fractions. On chromatography of the phenolic fractions from these experiments in

TABLE I

Distribution of Tritium in Metabolites of Testosterone-1,2-^{3}H

Expt	Fraction dpm x 10=5*			Aromatization Phenolic + H_2O x 100%
	Neutral	Phenolic	Water	Total
1	2921	326	-	10.4
2	30.4	2.6	2.7	15.5
3	30.1	3.4	2.4	16.1
4	126.0	24.0	39.0	33.2
5	162	37.0	39.0	31.8

*Data of Experiment 1 are units of area in scans of chromatograms.

Substrate: 2 μc testosterone-1,2-^{3}H + 1 gm of tissue in 15 ml. of Ringer phosphate buffer, pH 7.4, except in Experiments 4 and 5: 10 μc/vessel.

System II, the majority of the radioactivity was found to migrate with authentic 2-methoxyestrone.

Identification of 19-norandrost-4-ene-3,17-dione (Experiment 3). In each of six vessels, one gram of prostate slices was incubated with a mixture of 6 μc 1,2-^{3}H-testosterone (30 c/mmole) and 15 μc 4-^{14}C-19-nortestosterone (23.2 μc/μmole). Following a 90 min. incubation, deproteinization and extraction, the alkali-washed extract was chromatographed in System I. From this chromatogram, the radioactive material associated with added 5α-androstane-17β-ol-3-one was eluted, mixed with 19-norandrost-4-ene-3,17-dione, and chromatographed in the Bush A system which partially separated the two standards and associated activity. That part of the Bush chromatogram containing the UV-absorbent 19-norandrost-4-ene-3,17-dione standard was eluted and mixed with 1 mg. authentic standard. On gradient elution from an Alumina III column, the product was obtained in tubes 54-66 with almost constant specific activity of both ^{14}C and ^{3}H. The eluate contained 1.88% of the tritium and 1.38% of the ^{14}C of the substrate. After adding 30 mg. of standard, the pooled eluate was recrystallized to constant specific activity from dilute acetone, converted to the dioxime by the procedure of Calvin et al. (21), recrystallized from aqueous methanol, returned to the free form by exchange with pyruvate, and recrystallized to constant specific activity (Table 2). Thus, of 0.2 nanomole testosterone and 647 nanomoles 19-nortestosterone, .0037 and 89.28 nanomoles, respectively, were converted to 19-norandrost-4-ene-3,17-dione by one gram of tissue.

Identification of 19-nor-5α-androstane-17β-ol-3-one (Experiment 4). Six one-gram lots of tissue were each incubated with 3 μc, 1,2-^{3}H-testosterone (30 c/mmole). The alkali-washed extracts of each vessel were

TABLE 2

Identification of 19-norandrost-4-ene-3,17-dione
(Experiment 3)

Product	Specific Activity of Crystals (dpm/μmole)												$^3H/^{14}C$
	^3H						^{14}C						
	Calculated	n-3	n-2	n-1	n	Mean - S.E.	Calculated	n-3	n-2	n-1	n	Mean - S.E.	
free*	40.7	42.3	30.9	51.7	25.6	37.6 - 11.7	70.2	73.7	50.5	88.5	44.9	64.4 - 64.3	0.58
dioxime		40.3	39.1	41.9	39.1	40.1 - 1.3		68.7	67.4	72.4	67.5	68.9 - 2.3	0.57
cleaved dioxime			41.1	39.5	43.5	41.4 - 2.0			70.2	68.0	75.0	71.0 - 3.6	0.58

Six gms tissue each incubated 90 minutes at 37° with 0.19 nanomole 1,2-^{3}H-testosterone (30 mc/μmole) and 1.011 μmole 4-^{14}C-19-nortestosterone (23.2 μc/μmole). *Balance out of adjustment.

chromatographed in System I. From the leading edge of the testosterone-containing area of these chromatograms, 19-nor-5α-androstane-17β-ol-3-one was resolved in the Bush B-3 system and found to contain 15% of the total activity of the neutral fraction. To further purify and identify this product, it was taken from the paper and applied to an alumina III column. On gradient elution it emerged as a single symmetrical peak in tubes 39-60. A portion of the eluate from the column was diluted with standard to a specific activity of 9.42×10^3 dpms. per micromole and chromatographed on a thin layer of silica gel with cyclohexane-ethyl acetate (1:1). The radioactivity moved with the authentic standard at an R_f of 0.39. Of the zone eluted from the plate, one-half was oxidized with chromic acid to 19-nor-5α-androstane-3,17-dione, the other half was reduced with sodium borohydride to 19-nor-5α-androstane-3β, 17β-diol. Each of these derivatives was chromatographed on a thin layer plate adjacent to the appropriate standard. On both plates the mobility of the derivative was identical to the standard. The specific activity of the oxidation product was 8.62×10^3 dpms. per micromole, of the reduction product 8.60×10^3 dpms. per micromole.

The remainder of the eluate from the gradient elution column was diluted with 50 mg. 19-nor-5α-androstane-17β-ol-3-one and recrystallized from 50% acetone to constant specific activity. The homogeneous crystals were pooled, acetylated, mixed with 6.5 mg. 19-nor-5α-androstane-17β-acetoxy-3-one and recrystallized to constant specific activity from dilute ethanol. Following saponification of the crystals in 0.2% Na_2CO_3, they were again recrystallized from aqueous acetone. Table 3 shows that the specific activities of crystals of the acetate and of the free compound obtained following saponification agreed with the

TABLE 3

Identification of 19-nor-5α-androstane-17β-01-3-one
(Experiment 4)

Product	Specific Activity of Crystals (dpm/μmole)				
	Calculated	n-2	n-1	n	Mean
Acetate	375	380	315	423	373 ± 54.4
Saponified acetate	-	387	397	395	393 ± 5.2

Six gms tissue each incubated 90 minutes at 37° with 600 nanomoles 1,2-^{3}H-testosterone (30 mc/μmole).

calculated value indicating the homogeneity of the product obtained from the Bush chromatogram. Thus, in this experiment, one gram portions of prostate catabolized 0.1 nanomole of 1,2-^{3}H-testosterone to 0.015 nanomole of tritiated 19-nor-5α-androstane-17β-ol-3-one.

Identification of 2-methoxyestrone (Experiment 5). Three vessels, each containing 1 gm. of tissue slices and 3 μc 1,2-^{3}H-testosterone (30 c/mmole), were incubated 90 min., deproteinized and extracted as usual. Thirty five percent of the recovered radioactivity was in the phenolic fraction. This fraction from each vessel was supplemented with 200 μg. 2-methoxyestrone and chromatographed in System II. The 2-methoxyestrone eluates from these strips constituting 28.7% of the total phenolic material or 10% of the total recovered activity were pooled, supplemented with 200 μg. authentic standard and chromatographed in System III to give a single peak of radioactivity. Another 200 μg. of standard was added to the eluate from the papergram before acetylation and thin layer chromatography with cyclohexane-ethyl acetate (1:1) (R_f .625, in agreement with standard). The eluted 2-methoxyestrone-3-

TABLE 4

Chromatographic Identification of Isolated 2-Methoxyestrone
(Experiment 5)

Procedure	Product	Specific Activity (dpm x 10^{-4}/μmole)
PC (System III)	2-methoxyestrone	8.94
Add carrier		6.44 (calculated)
Acetylate TLC (CyHex:Et.Ac)	2-methoxyestrone-3-acetate	6.63
Reduction, TLC (B_2-Et.Ac)	2-methoxyestradiol-17β-3-acetate	6.89

CyHex:Et.Ac. = Cyclohexane:Ethyl Acetate (50:50)
B_2-Et.Ac. = Benzene:Ethyl Acetate (80:20)
TLC = Thin layer chromatography
Three gms tissue each incubated 90 minutes at 37° with 3 μc 1,2-^{3}H-testosterone (30 c/mmole).

acetate was reduced with $NaBH_4$ in dioxane to 2-methoxyestradiol-3-acetate and subjected to thin layer chromatography with benzene-ethyl acetate (4:1) (R_f 0.81, in agreement with standard.) Table 4 shows the stability of the specific activity of the product through derivatization and chromatography. Approximately 7.5% of the tritium of the substrate was incorporated into 2-methoxyestrone. Correcting for the 50% loss of tritium from this substrate during aromatization (21) the experiment shows that one gram of tissue converted 15% (0.015 nanomole) of 0.1 nanomole of 1,2-^{3}H-testosterone to 2-methoxyestrone in 90 minutes.

Metabolic routes for aromatization of testosterone (Experiment 6). In each of six vessels one gram of tissue slices was incubated 90 minutes with a mixture of 3 μc 1,2-^{3}H-testosterone (30 c/mmoles) and 0.3 μc 4-^{14}C-19-nortestosterone (23.2 μc/μmole). The 19-nortestosterone and

19-nor-5α-androstane-17β-ol-3-one were resolved from testosterone and 19-norandrost-4-ene-3,17-dione was separated from the 5α-androstane-17β-ol-3-one in the Bush B-3 and A systems, respectively. The 19-nortestosterone, further purified by thin layer chromatography with ethyl acetate-cyclohexane (1:1), acetylation, and thin layer chromatography with benzene-ethyl acetate (4:1), was found to contain 2.6% of the tritium and 12.8% of the ^{14}C of the substrate. The calculated specific activities of the acetate were ^{3}H = 281.9 dpm/μg, ^{14}C = 132.0 dpm/μg; found ^{3}H = 302 dpm/μg, ^{14}C = 186 dpm/μg.

Gradient elution of the 19-nor-5α-androstane-17β-ol-3-one, mixed with two mg. authentic standard, evolved a homogeneous peak, fractions 39-60, which reacted positively with m-dinitrobenzene-potassium hydroxide, displayed no absorbence in the ultraviolet and contained approximately 15% of the total tritium and 3.4% of the ^{14}C of the substrate. This product was not further characterized since it is not considered an intermediate in the aromatization of testosterone.

The 19-norandrost-4-ene-3,17-dione from the Bush A chromatogram was subjected to gradient elution. A single radioactive, ultraviolet-absorbent peak was chromatographed vs. the authentic diketone on a thin layer plate. After reduction of the steroid with sodium borohydride, it was chromatographed on a thin layer vs. 19-norandrost-4-ene-3β,17β-diol. The eluted reduction product was oxidized and chromatographed, as before, vs. 19-norandrost-4-ene-3,17-dione (Table 5a). In each of these chromatograms, the radioactive material moved at the same rate as the adjacent reference standard. Of the added substrate 1.25% of the tritium and 19.8% of the ^{14}C appeared in this product.

From the alkali-soluble fraction, 2-methoxyestrone was isolated

TABLE 5a

Chromatographic Identification of 19-norandrost-4-ene-3,17-dione (Experiment 6)

Procedure	Product	Specific Activity (dpm/μg)		$^{3}H/^{14}C$ (dpm/dpm)
		^{3}H	^{14}C	
Calculated	column eluate	125.8	66.3	1.89
TLC	19-norandrost-4-ene-3,17-dione	155.0	113.0	1.37
Reduce, TLC	19-norandrost-4-ene-3β,17β-diol	138.0	115.0	1.20
Oxidize, TLC	19-norandrost-4-ene-3,17-dione	141.5	100.0	1.41

Six gms tissue each incubated 90 minutes at 37° with 3 μc 1,2-^{3}H-testosterone (30 c/mmole) and 0.3 μc 4-^{14}C-19-nortestosterone (23.2 μc/μmole).

TABLE 5b

Chromatographic Identification of 2-Methoxyestrone (Experiment 6)

Procedure	Product	Specific Activity (dpm/μg)		$^{3}H/^{14}C$ (dpm/dpm)
		^{3}H	^{14}C	
PC (Mahesh I)	MeOE	916	19.4	47.2
Add carrier, PC	"	723	13.3	54.5
PC (System III)	"	515	9.2	55.7
Acetylate, TLC (CyHex-Et.Ac.)		544	10.8	50.2

through successive chromatography System II, System I of Mahesh and System III. The product was acetylated and found by thin layer chromatography with cyclohexane-ethyl acetate (1:1) to move with authentic 2-methoxyestrone-3-acetate (Table 5b). Correcting for the 50% loss of tritium accompanying aromatization, 16.3% of the testoster-

one and 2.25% of the 19-nortestosterone of the substrate were incorporated into the estrogen.

The data from all the experiments is presented in Table 6 in order to show the actual molar conversions of the substrates to the various products.

DISCUSSION

It has been shown that the human prostate metabolizes testosterone to a variety of estrane derivatives, including 19-nortestosterone, 19-norandrost-4-ene-3,17-dione, 19-nor-5α-androstane-17β-ol-3-one and 2-methoxyestrone, without benefit of any exogenous cofactors. The tissue also attacks 19-nortestosterone to produce 19-norandrost-4-ene-3,17-dione, 19-nor-5α-androstane-17β-ol-3-one and 2-methoxyestrone.

One of the primary objectives of this study was to determine the metabolic pathways traversed by the carbon of testosterone in the biosynthesis of these estranes. It was anticipated that, as in placental preparations, the principal route to estrogens was direct, via 19-OH- and 19-oxo-testosterone, but that 19-nortestosterone and/or 19-norandrost 4-ene-3,17-dione could be aromatized also and could serve as intermediates in the conversion of testosterone to estrogen. The results confirm these expectations: The isolation of dual-labelled 19-nortestosterone and 19-norandrost-4-ene-3,17-dione shows that testosterone is 10-demethylated, in confirmation of our earlier report (3). The diketone of Experiment 5 has about one-third the $^{3}H/^{14}C$ of the 19-nortestosterone. This is probably due to concurrent 17β-dehydrogenation of the 19-nor substrate and of the dual-labelled 19-nortestosterone. The presence of both ^{3}H and ^{14}C in the 2-methoxyestrone provides evidence that the prostate aromatizes both testosterone and 19-nortes-

TABLE 6

Metabolic Activity of One Gram of Prostate

Product	Experiment	Substrate	Quantity (mmols/g. tiss.)	%	(mmols/gm. tiss.)	(mmols/mmole substrate)
19-nortestosterone	6	T	0.1	2.6	.0026	.026
		Nor-T	12.9	12.8	1.6	.127
19-norandrost-4-ene-3,17-dione	3	T	0.2	1.9	.0037	.0185
		Nor-T	647.0	13.8	89.23	.138
	6	T	0.1	1.25	.00125	.0125
		Nor-T	12.9	19.8	2.48	.192
19-nor-5α-androstane-17β-ol-3-one	4	T	0.1	15.0	.015	.150
	6	T	0.1	15.0	.015	.150
		Nor-T	12.9	3.3	.427	.033
2-Methoxyestrone	5	T	0.1	15.0	.015	.150
	6	T	0.1	16.6	.0166	.166
		Nor-T	12.9	2.25	.2902	.023

T = testosterone; Nor-T = 19-nortestosterone; nmole = nanomole. See test and previous tables for experimental details.

tosterone. However, the high $^{3}H/^{14}C$ ratio of the estrogen, as compared to the 19-norsteroids, indicates that the extent of metabolism of testosterone via the 4-estrenes is small relative to that which bypasses these compounds.

Conversion of 15% of the testosterone to 19-nor-5α-androstane-17β-ol-3-one was expected in view of our earlier observations of rapid 4,5-reduction of testosterone to 5α-androstane-17β-ol-3-one by both rat and human prostate (23). While there is significant incorporation of ^{14}C from 19-nortestosterone into this compound, the $^{3}H/^{14}C$ ratio indicates that most of the saturated compound arises from testosterone by a pathway which does not include 19-nortestosterone. It presently appears most likely that this steroid is predominantly the product of 10-demethylation of 5α-androstane-17β-ol-3-one. Preliminary incubations of 1,2-^{3}H-5α-androstane-17β-ol-3-one seem to confirm this. Due to the unavailability of the 19-nor-17-ketosteroids which Engel et al. (24) recovered following parenteral administration of 19-nortestosterone, no attempt could be made to determine if these compounds are products of testosterone metabolism in the prostate.

Of the estrogens, only 2-methoxyestrone has been rigorously identified. An examination of the scans of chromatograms of the alkali-soluble material shows some radioactivity having the mobility of 2-methoxyestradiol-17β or estrone and a regularly-appearing peak at the origin, confluent with estriol or 2-methoxyestriol. In no experiment has there been any evidence of either estradiol-17β or -17α. Exhaustive attempts with thin layer chromatography and recrystallization have yielded tentative identification of 2-methoxyestradiol-17β, but none of estrone, estriol or epiestriol. In preliminary trials, in which

25 μg of estrone or 17β-estradiol was added to the 1,2-^{3}H-testosterone of the substrate, there was no evident dilution of the specific activity of 2-methoxyestrone or accumulation of any activity in either of the added estrogens. Yet both we (25), employing 6,7-^{3}H-estradiol-17β as substrate, and the San Antonio group (26), using 16-^{14}C-estrone, have demonstrated 17β-dehydrogenase, 2β-hydroxylase and O-methyl transferase activity in human prostate. We are led to suspect that there is methoxylation of some estrogen precursor, perhaps 2-OH-testosterone.

The physiological significance of these findings must also be considered:

Segaloff (4) has proposed that testosterone may be a proandrogen - a precursor to active 19-nortestosterone. As previously discussed (3), if this proposal is true, the biological activity of testosterone can be attributed to and is proportional to the extent of demethylation. Segaloff observed the 10-fold greater androgenicity of 19-nortestosterone in his parenteral assays only when the steroid bore methyl groups at the 7α- and 17α-positions. He interpreted the synergistic effect of these substituents on the 19-nortestosterone "as indicative of sufficient protection to permit delivery of the highly active 19-norsteroid to the end organ" (27). If there is no suppressive or enhancing effect of the methyl groups on the biological activity in the target organ, Segaloff's data would indicate that, biologically, 1 mole of 19-nortestosterone is equal to 10 moles of testosterone. In the present metabolic studies, however, it is evident that, biochemically, 100 moles of testosterone is metabolized to somewhat more than the 2.6 moles of 19-nortestosterone recovered, since the 19-norandrost-4-ene-3,17-dione

and some part of 2-methoxyestrone and the 19-nor-5α-androstane-17β-ol-3-one appeared to arise from 19-nortestosterone also. More data would be needed to determine if the 10:1 ratio of biological activities of the two steroids, 19-nortestosterone and testosterone, respectively, are accountable to one mole of 19-nortestosterone arising from ten moles of testosterone.

This is the first known report of aromatization of testosterone by prostatic tissue. That only the extremely low potency (12) estrogen, 2-methoxyestrone was conclusively isolated is probably due to the equilibrium of the estradiol-17β-dehydrogenase favoring the ketonic form (Fishman found this tendency (28) and Engel (24) recovered only estrone in the estrogen metabolites of 19-nortestosterone), an active 2β-hydroxylase (25, 26), and a ready conversion of estrone to 2-methoxyestrone, in vitro (25, 26). Failure to identify 2-OH-estrone in the present study may be accountable to the lability of this compound (29), to the high efficiency of ether formation by the O-methyl transferase, or, perhaps, to methoxylation of an estrogen precursor (1).

Woodruff and Perez-Mesa have shown a need for estrogen as well as androgen to maintain the normal morphology of the rat prostate (30). Whether the normal prostate of man has similar requirements is not known. If it does, current efforts in our laboratory to compare testosterone metabolism in normal, benign hypertrophic and carcinomatous prostate may indicate whether or not the gland meets its estrogen requirement by aromatization at the observed rate or depends upon circulating estrogen. The estrogenized appearance of the benign hypertrophic gland (31) may reflect a self-intoxication due to

excessive conversion of androgen to estrogen per se, to an increased sensitivity to estrogen, or at the expense of depletion of its concentration of androgen.

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REFERENCES

1. Acevedo, H. F., and Goldzieher, J. W., BIOCHIM. BIOPHYS. ACTA, 97, 564 (1965).
2. Chamberlain, J., Jagarinec, N., and Ofner, P., BIOCHEM. J., 99, 519 (1966).
3. Farnsworth, W. E., STEROIDS, 6, 519 (1965).
4. Segaloff, A., STEROIDS, 1, 299 (1963).
5. Wilds, A. L., and Nelson, N. A., J. AM. CHEM. SOC., 75, 5366 (1953).
6. This product was also prepared by the method of Bowers, A., Halsall, T. G., Jones, E. R. H., and Lemin, A. J., J. CHEM. SOC., 2548 (1953) with identical physical properties.
7. Neudert, W., and Ropke, H., ATLAS OF STEROID SPECTRA, Springer-Verlag, New York 1965.
8. Hartman, J. A., Tomasewski, A. J., and Dreiding, A. S., J. AM. CHEM. SOC., 78, 5662 (1956).
9. Bowers, A. Ringold, H. J., and Denot, E., J. AM. CHEM. SOC., 80, 6115 (1958).
10. Djerassi, C., Cais, M., and Mitscher, L. A., J. AM. CHEM. SOC., 81, 2386 (1959).
11. Chen, C., TETRAHEDRON, 3, 43 (1958).
12. Krachy, S., and Gallagher, T. F., J. BIOL. CHEM., 229, 519 (1957).
13. Fishman, J., J. AM. CHEM. SOC., 80, 1213 (1958).
14. Bush, I. E., BIOCHEM. J., 50, 370 (1952).
15. Mahesh, V. B., STEROIDS, 3, 647 (1964).
16. Axelrod, L. R., J. BIOL. CHEM., 205, 173 (1953).
17. Allen, W. M., J. CLIN. ENDOCRINOL., 10, 71 (1950).
18. Baggett, B., Engel, L. L., and Fielding, L. L., J. BIOL. CHEM., 213, 87 (1955).
19. Axelrod, L. R., J. BIOL. CHEM., 203, 59 (1953).
20. Bray, G. A., ANAL. BIOCHEM., 1, 279 (1960).
21. Calvin, H. I., Van de Wiele, R. L., and Lieberman, S., BIOCHEMISTRY, 2, 648 (1963).
22. Axelrod, L. R., and Goldzieher, J. W., J. CLIN. ENDOCRINOL., 22, 537 (1962).

23. Farnsworth, W. E., NATIONAL CANCER INSTITUTE MONOGRAPH, 12, 323, (1963).
24. Engel, L. L., Alexander, J., and Wheeler, M., J. BIOL. CHEM., 231, 159 (1958).
25. Farnsworth, W. E., unpublished.
26. Acevedo, H. F., and Goldzieher, J. W., BIOCHIM. BIOPHYS. ACTA, 97, 571, (1965).
27. Reference 4, p. 313
28. Fishman, J., Bradlow, H. L., and Gallagher, T. F., J. BIOL. CHEM., 235, 3104 (1960).
29. Fishman, J., J. CLIN. ENDOCRINOLOGY, 23, 207 (1963).
30. Woodruff, M. W., and Perez-Mesa, C., J. UROL., 88, 273 (1962).
31. Brandes, D., Kirchein, D., and Scott, W. W., LAB. INVEST., 13, 1541 (1964).