ACETYLATION AND HYDROXYLATION OF 5α-ANDROSTANE-3β,17β-DIOL BY PROSTATE AND EPIDIDYMIS

Peter Ofner and Richard L. Vena

Steroid Biochemistry Laboratory of the Medical Services, Lemuel Shattuck Hospital; Department of Urology, Tufts University School of Medicine and Departments of Pharmacology, Harvard School of Dental Medicine and Harvard Medical School, Boston, Massachusetts 02130, U.S.A.

and

Robert F. Morfin

Laboratoire de Biochimie, Faculté de'Médecine, B.P. 815, Brest, France

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ABSTRACT

An unknown radiometabolite, formed in the canine prostate and epididymis after intra-arterial infusion of testosterone-4-^{14}C in physiologic saline and extraction of the organs with ethyl acetate-acetone, was identified as the 3-monoacetate of 5α-androstane-3β,17β-diol (3β-diol). Transformation of 3β-diol-^{14}C to its identified 3-monoacetate derivative could also be demonstrated, if the incubation of the radiosubstrate with minced canine prostate was terminated by ethyl acetate extraction. The formation of polar products in high yield was noted. Whereas minced canine prostate actively converted 5α-androstane-3α,17β-diol-^{14}C to 17β-hydroxy-5α-androstan-3-one-^{14}C, the same preparation hydroxylated 3β-diol ^{14}C predominantly at the 7ξ- and, to a lesser extent, at the 6ξ-positions. Partial identification of the hydroxylated radiometabolites was by crystallization of the CrO_3-oxidation products 5α-androstane-3,6,17-trione-^{14}C and 5α-androstane-3,7,17-trione-^{14}C to constant SA and by GLC/MS of the latter derivative. NADPH-supplementation of the preparation enhanced the yield of hydroxylated products derived from 3β-diol-^{14}C in a 1 hr incubation from 22% to 41%. Analogous supplemented incubations of benign hyperplastic human prostate and canine epididymis produced polar metabolites (in 12.5% and 76% yields, respectively) which gave rise to similar proportions of the same androstanetrione epimers on CrO_3-oxidation.

INTRODUCTION

On infusion of testosterone-4-^{14}C into the median sacral artery of dogs, we encountered unknown transformation products of high and low chromatographic mobilities in the ethyl acetate-acetone extracts of the prostate and epididymis (1,2). There was a particular need to identify metabolite(s) isopolar with androstenedione (4-androstene-3,17-dione), since the product(s) accounted for a substantial share of the prostatic radioactivity (1) and predominated in the epididymal extract (2). Identification of the 3-monoacetate of 5α-androstane-3β,17β-diol (3β-diol) as a major radiometabolite migrating with androstenedione on silica-gel chromatography and experiments which assign to ethyl acetate the role of acetate donor *in vivo* and *in vitro* were described in a preliminary communication (2).

Study of the transformation of 5α-androstane-3α,17β-diol (3α-diol) and 3β-diol in minced canine and human tissue, initiated as a result of the infusion experiments, led to the recognition of the high substrate effectiveness of the 3β-hydroxy epimer in prostatic and epididymal hydroxylation reactions. This paper describes the aforementioned findings in detail and reports the partial identification of 5α-androstane-3β,7ξ,17β-triol and 5α-androstane-3β,6ξ,17β-triol as the major and a minor hydroxylation product.

MATERIALS AND METHODS

Testosterone-4-^{14}C (54-58 mCi/mmole) was purchased from New England Nuclear Corp. 4-^{14}C-Labeled 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one), 3α-diol and 3β-diol were prepared biochemically from testosterone-^{14}C (3).

All solvents were redistilled before use; acetone and ethyl acetate were stored over anhydrous sodium carbonate for 2 days before distillation from that chemical.

Thin-layer chromatography. TLC systems were: (1) benzene-ethanol (9:1 v/v) on Merck silica gel G (2) two-dimensional: chloroform-ether (9:1) 2X/chloroform-ether (7:3) 1X on silica gel G (3) benzene-ethanol (97:3) on Merck alumina G (4) methylene chloride:ether (9:1) on alumina G.

5α-Androstane-3β,17β-diol 3-monoacetate, mp 117-119° (Ref. 4 gives mp 114-116°), was purchased from Steraloids Inc., Pawling, N.Y. It was also prepared as a reference specimen for examination by GLC/MS as described (5). It proved homogenous on TLC with system 1, developed 1X.

3β-Hydroxy-5α-androstane-7,17-dione (NSC-37849) and 17β-hydroxy-5α-androstane-3,6-dione (NSC-59613) were donated by the Experimental Biology Projects Section, National Cancer Institute. Oxidation with CrO_3 in acetone converted the former steroid into 5α-androstane-3,7,17-trione (7-trione) in 75% yield; after preparative TLC with two developments in solvent system 1 and crystallization from acetone-water, the mp of the trione was 234-235° (Ref. 6 gives mp 237-239°). The latter precursor was similarly oxidized to 5α-androstane-3,6,17-trione (6-trione) in 34% yield; after identical purification the mp of this product was 196-197° (Ref. 7 gives mp 191-192°). Mass spectra of these epimers have been recorded (8).

Infusion and incubation conditions. Testosterone-^{14}C in physiologic saline was infused through a catheter placed in the median sacral artery of a mature dog as described previously (1). The radiosteroid reached the prostate gland via the urogenital arteries and the epididymis via the caudal vesical and deferential arteries. Since the epididymis is supplied by branches from both the deferential and internal spermatic arteries, the radioactivity found in the organ on infusion will be largely localized in the cauda and corpus epididymides. Spermatozoa were not removed prior to tissue extraction.

Prostate glands and epididymides, removed from mature dogs immediately after death by electrocution or from an overdose of pentobarbital sodium, and a fresh specimen of human prostate with benign hyperplasia obtained at suprapubic prostatectomy were chilled over cracked ice, blotted several times on surgical gauze and minced at 4° by cutting with scissors. The incubation mixtures consisted of 5 ml of 67 mM phosphate buffer (pH 7.4), 2.43 nmoles of 4-^{14}C-labeled substrate and 200 mg tissue mince in the presence or absence of 1.5 mg NADPH. Incubations were for 1 hr in air at 37°. Enzymatic action was stopped by shaking the mixtures vigorously with 6 ml of either ethyl acetate or methylene chloride:acetone (4:1 v/v) and subsequent storage at -17°. In preparative incubations the quantities of constituents were increased 10 fold. Examination for bacterial contamination showed either no growth or the presence of less than 10^4 colonies.

Extraction and isolation of radiosteroids. Procedures for preparing, purifying and resolving ethyl acetate-acetone extracts of the infused organs and solvent extracts of incubation mixtures have been described (1,9). In the present study, the only modification in processing the extracts of whole prostate glands and of preparative

incubation mixtures was to substitute hydrophobic gel chromatography for the 2 steps of lipid removal by partitioning between n-hexane and 80% methanol and TLC of the purified extracts with chloroform on silica gel G. The dry initial extracts, prepared by removal of solvent and azeotropic distillation with absolute ethanol at 40° in a stream of N_2 under reduced pressure, were left in 20 ml methanol overnight. The ethyl acetate solution of the methanol-insoluble residue contained only 2-5% of the total radioactivity. The methanol extract was freed of solvent and chromatographed with trimethylpentane/isopropanol/H_2O (80:400:120) and azulene as a column marker on hydroxyalkoxypropyl-Sephadex (10,11). The column fraction collected after removal of a compact yellow brown band of interfering lipid until just before elution of the azulene marker contained 90-95% of the applied radioactivity. TLC of this fraction with double development in system 1 could then be readily accomplished without interference of residual lipid. Endogenous cholesterol which persisted in radioactive eluates of the zone containing androstenedione and the unknown radiometabolite(s) was removed prior to examination by GLC/MS using the delipidation procedure of Ismail et al. (12).

Derivative formation. Portions of the initial extract and of chromatographic fractions were oxidized with CrO_3 in acetone, acetylated with acetic anhydride in pyridine or converted to the trimethylsilyl ether (TMSE) derivatives (1,5). Suspected radiosteroid acetates were hydrolyzed by storing in 10 ml of 0.15% sodium hydroxide in 80% methanol under N_2 at room temperature overnight. The solution was then diluted with an equal volume of water and the chloroform extract washed with 5% hydrochloric acid and water. The oxidized, acetylated and hydrolyzed fractions were purified and resolved by TLC. TMSE derivatives were analyzed by GLC and GLC/MS (5).

Determination of SA. Radioactivity measurements in the liquid scintillation spectrometer and carrier-weight analysis by GLC for SA determination were carried out as reported (1,3). Sufficient counts were allowed to accumulate so that the counting error did not exceed ± 1%.

Crystallization to constant SA. The homogeneity of the radiometabolites was examined by crystallization with authentic carrier from the following solvent systems: methanol/water (M/W); acetone/water (A/W); ether/n-hexane (E/H); ethyl acetate/n-hexane (EA/H). Redistribution of SA of the mother liquor fractions, based on the weight of crystals, permits direct comparison of the SA of crystals and mother liquors and is calculated as described (3).

Gas liquid chromatography-mass spectrometry. GLC/MS was carried out with the double beam AEI model MS-30 gas chromatograph/mass spectrometer fitted with a membrane molecular separator. Details of the analysis of epimeric androstanediol monoacetates have been described (5). A glass column 9 ft. long containing 1.63% fluorosilicone QF-1 on 100-200 mesh gas-chrom Q as stationary phase was used at 237° with helium as carrier gas to separate 7-trione from 6-trione; retention indices were 41.25 and 42.25, respectively. MS

conditions for the analysis of the epimeric triones were: temperatures of the molecular separator, ion source and line 210°, 200° and 200°, respectively; energy, 24 eV; scanning at 3 sec/decade and a resolution of 1000.

RESULTS

Identification of a radiometabolite of intra-arterially infused testosterone-4-^{14}C in ethyl acetate-acetone extracts of canine prostate and epididymis as 5α-androstane-3β,17β-diol 3-monoacetate.

Unidentified canine prostatic radiometabolite(s) had been shown to be isopolar with androstenedione on silica gel G with single development in chloroform-ether (7:3 v/v), but to be readily separated from it with TLC system 4 (1). Acetylation or CrO_3-oxidation effected complete transformation of the unknown to products with the chromatographic mobilities of diacetates or monoketone monoacetates, respectively.

Following a 10-minute intra-arterial infusion (1) of testosterone-^{14}C (9.74 μCi/51.2 μg) in physiologic saline, both androstenedione-^{14}C and the unknown radiometabolite(s) (Unknown 1) could be isolated (TLC system 1) from delipidated prostatic and untreated epididymal extracts derived from ethyl acetate-acetone (1:1 v/v) homogenates of the benign hyperplastic prostate gland (50.5 g) and epididymides (8.7 g) of dog I. The prostatic TLC fraction accounted for 13.5% of the radioactivity (1,780,000 dpm) extracted from the organ and was resolved with TLC system 4 into 11.9% androstenedione and 88.1% unknown radiometabolite(s). The corresponding epididymal TLC fraction contained 19.9% of the extracted radioactivity (212,000 dpm) and consisted of 2.0% androstenedione and 98.0% unidentified constituent(s).

(i) Alkaline hydrolysis. Hydrolysis of prostatic Unknown 1 with

methanolic alkali as described gave rise to a mixture of 3α-diol and 3β-diol (46% yield) which was resolved by TLC with double development in system 3. Table 1 presents evidence of the identity of the hydrolysis products. The ratio of 3β/3α-hydroxysteroid epimers was 5.1.

TABLE 1. CRYSTALLIZATION OF RESOLVED HYDROLYSIS PRODUCTS OF UNKNOWN PROSTATIC RADIOMETABOLITES

CARRIER	NO.	SYSTEM†	STARTING*	CR	MLQ
3α-diol	1	M/W	62 (1060)	56	65
3β-diol	1	M/W	95 (5700)	95	94
	2	A/W		95	94

†cf. Materials and Methods; *SA of starting material, DPM in parenthesis; CR = SA of crystals; MLQ = redistributed SA of mother liquor (3).

All SA data in Tables 1, 2 and 5 are given as dpm/μmole.

(ii) Crystallization with 3β-diol 3-monoacetate carrier to constant SA. Portions of the prostatic fraction were crystallized with carrier before and after chromatographic separation of androstenedione-^{14}C. Because of its low androstenedione-^{14}C content, epididymal Unknown 1 was not separated before crystallization. The data listed in Table 2 show that 3β-diol-^{14}C 3-monoacetate constituted 53.8% of the unresolved and 63.4% of the purified prostatic TLC fraction - in satisfactory agreement with the chromatographic analysis - and comprised 27% of the epididymal fraction under investigation. The prostatic and epididymal radiometabolite accounted for 7.6% and 5.3% of the radioactivity found in the respective organs, compared with 9.7% and 18.0% of unesterified 3β-diol (2).

TABLE 2. CRYSTALLIZATION OF UNKNOWN RADIOMETABOLITES WITH 5α-ANDROSTANE-3β,17β-DIOL 3-MONOACETATE CARRIER

ORIGIN OF FRACTION	CRYSTALLIZATION NO.	CRYSTALLIZATION SYSTEM[+]	SPECIFIC ACTIVITY STARTING[*]	SPECIFIC ACTIVITY CR	SPECIFIC ACTIVITY MLQ[‡]
CANINE PROSTATE IN VIVO	1	M/W	484 (47,400)	334	621
	2	A/W		293	373
	3	E/H		267	297
	4	M/W		260	270
CANINE PROSTATE IN VIVO AFTER SEPARATION OF ANDROSTENEDIONE	1	M/W	353 (34,600)	236	459
	2	A/W		225	239
	3	E/H		224	219
CANINE PROSTATE IN VITRO	1	M/W	565 (33,900)	549	575
	2	A/W		531	557
	3	E/H		519	558
	4	M/W		515	520
CANINE EPIDIDYMIS IN VIVO	1	M/W	206 (20,200)	75	332
	2	A/W		64	87
	3	E/H		59	68
	4	A/W		55	62

[+]cf. Materials and Methods; [*]DPM in parenthesis; CR = crystals; [‡]MLQ = redistributed SA of mother liquor (3).

(iii) Comparative solvent extraction. The prostate of dog II was cut along its median raphe following intra-arterial administration of testosterone-^{14}C (9.91 μCi/49.6 μg) as described (1). One of the resulting prostate specimens (15.3 g) and one epididymis (4.6 g) were separately extracted with chilled acetone, while the other prostate specimen (14 g) and epididymis (5.8 g) were separately treated with ice-cold ethyl acetate:acetone (1:1 v/v). When acetone was the extracting solvent, radioactive product migrating with 3β-diol 3-monoacetate on TLC was barely detectable, amounting to 0.3% of the total prostatic (1,080,000 dpm) and 0.1% of the epididymal (344,000 dpm)

radioactivity. In contrast, with ethyl acetate:acetone extraction, 2.5% of the total prostatic (1,500,000 dpm) and 6.0% of the epididymal (529,000 dpm) radioactivity was found in the TLC zone isopolar with this metabolite.

(iv) Effect of solvent pre-treatment on the prostate preparation. Fresh canine prostate mince (25 g) was homogenized in 90 ml ethyl acetate-acetone (1:1 v/v) in a Waring Blendor for 15 min. A solution of a mixture (3 μCi/15.4 μg) of 3 parts of 3α-diol-^{14}C and 1 part of 3β-diol-^{14}C in 10 ml of the same solvent was then added to the mixture and homogenization continued for 15 min. The tissue extract was then prepared and fractionated. Conversion to product isopolar on TLC with the 3-monoacetates accounted for 0.57% of the 5α-androstanediol epimers

Characterization of "Unknown 2".

This fraction (2) of unknown prostatic products migrated beyond 5α-androstane-3,17-dione on TLC with system 1 and accounted for 5.3% of the radioactivity recovered from the gland of dog I. "Unknown 2" was isopolar with the CrO_3-oxidation products of "Unknown 1" (1). Hydrolysis with methanolic alkali produced a mixture of radiometabolites (30.9% yield) with the same chromatographic mobilities (system 1) as androsterone and isoandrosterone. The ratio of 3β/3α-hydroxysteroid epimers was 1.8.

Incubation of 5α-androstane-3α,17β-diol-4-^{14}C and 5α-androstane-3β,17β-diol-4-^{14}C with minced canine prostate.

The evidence for ethyl acetate-derived 3-monoacetylation of 3β-diol-^{14}C obtained in the *in vivo* experiments prompted us to study the effect of terminating with this solvent the incubation of the ^{14}C-labeled 5α-androstanediol epimers in the absence and presence of minced canine prostate tissue.

We established that vigorous agitation of the phosphate buffer solution of 3β-diol-^{14}C with ethyl acetate following incubation of the radiosteroid in the absence of the tissue preparation under the cited conditions yielded < 0.2% transformation to product isopolar with the monoacetate.

The radiometabolite patterns presented in Table 3 were obtained by extracting preparative incubation mixtures of the labeled 5α-androstanediol substrates and fresh minced prostate from a mature dog with ethyl acetate and subjecting the solvent extract to TLC analysis as described.

TABLE 3. RADIOMETABOLITE PATTERNS OF 5α-ANDROSTANE-3α,17β-DIOL AND 5α-ANDROSTANE-3β,17β-DIOL IN CANINE PROSTATE

STEROIDS	^{14}C-SUBSTRATE	
	3α-diol	3β-diol
Polar unknown	3.3	11.0+
5α-Androstane-3α,17β-diol	51.7	0.3
5α-Androstane-3β,17β-diol	0.6	72.3
Isoandrosterone	-	1.2
Androsterone	1.0	5.6*
5α-Dihydrotestosterone	39.0	
Unknown 1	2.0+	8.9+
5α-Androstane-3,17-dione	1.7	-
Unknown 2	0.7	0.8

Values are expressed as percentage contributions of the listed steroids to the total radioactivity recovered from the TLC plate.

+Examined by GLC/MS (cf. text). *Not resolved by TLC.

Incubation Conditions: 24.3 nmoles, 0.5 μM substrate/2 g tissue mince/hr.

The patterns show a significant difference in the metabolic path-

ways of the epimers. Whereas 3α-diol favored 5α-dihydrotestosterone formation, 3β-diol was mainly converted to unknown polar and non-polar metabolites.

Both substrates yielded a fraction which was isopolar with the "Unknown 1" identified in the infusion study. We previously reported that the TMSE derivative of the fraction produced by either androstanediol epimer gave the same GLC peak at the position expected for the TMSE of authentic 3β-diol 3-monoacetate (5), with no evidence for the presence of 3α-monoacetate; we also provided the mass spectra of the fractions associated with these GLC peaks which proved to be identical with the spectrum of the aforementioned reference compound (2).

The "Polar Unknown" radiometabolite(s) were oxidized with CrO_3 in acetone and the ethyl acetate extract of the reaction mixture was purified by 2-dimensional TLC (system 2). Autoradiography of the chromatogram revealed a major androstanetrione zone of radioactivity, its blurred lower edge suggesting the presence of another constituent. The eluate of this zone was examined by GLC/MS as described. The retention index of the only significant GLC peak was 41.4. The mass spectra of the unknown-peak fraction and of authentic 7-trione, shown in Fig. 1, are identical.

Comparative radiometabolite patterns of 5α-androstane-3β,17β-diol-^{14}C in minced canine and human prostate and epididymis and partial identification of hydroxylation products.

Confirmation of ethyl acetate-derived acetylation and extensive hydroxylation of 3β-diol-^{14}C is provided by the data listed in Table 4. Unknown 1 derived by ethyl acetate extraction of canine-prostate-mince incubate and TLC fractionation of the solvent extract was identified as

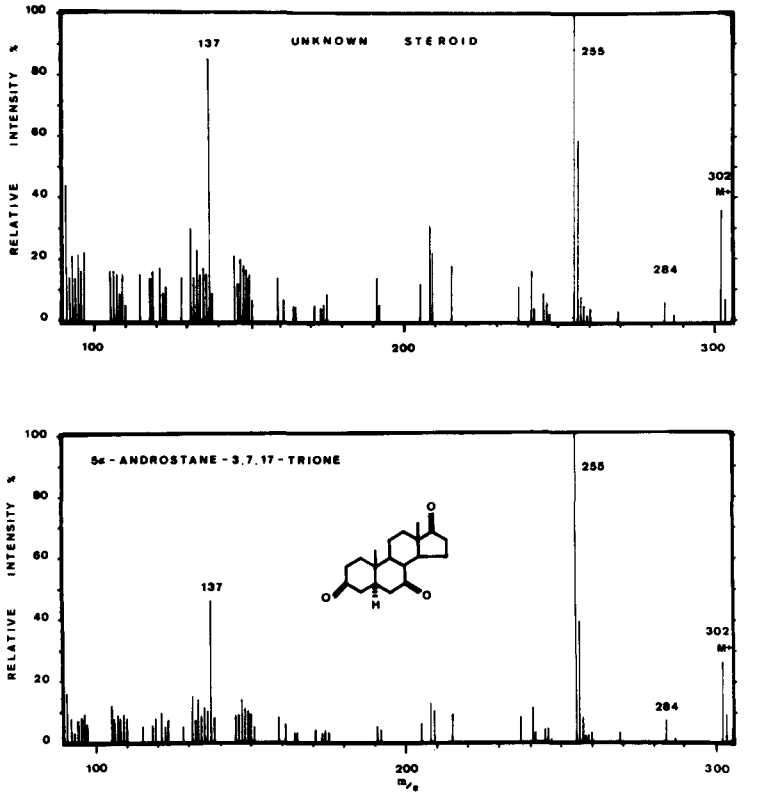

Figure 1. Mass spectra of CrO_3-oxidation product of "Polar Unknown" radiometabolite fraction (Table 3) and of 7-trione (cf. Materials) with GLC retention indices of 41.4 and 41.25, respectively. The spectra were recorded under identical conditions as described using a double beam AEI model MS-30 GLC/MS instrument.

3β-diol-^{14}C 3-monoacetate by crystallization with authentic carrier (Table 2).

TABLE 4. RADIOMETABOLITE PATTERNS OF 5α-ANDROSTANE-3β,17β-DIOL IN CANINE AND HUMAN HYPERPLASTIC PROSTATE AND CANINE EPIDIDYMIS

SUPPLEMENTATION	NONE	NONE	NADPH	NADPH	NADPH
TISSUE	CP	CP	HP	CP	CE
EXTRACTING SOLVENT	EA	Me/A	EA	Me/A	Me/A
STEROID PATTERN					
Polar unknowns	18.0*	22.0	12.5*	41.3	75.8
5α-Androstane-3β,17β-diol	73.9	74.1	80.5	50.4	20.0
Isoandrosterone	0.7	0.6	1.4	2.6	1.7
5α-Dihydrotestosterone	1.9	2.4	1.0	4.0	1.5
Unknown 1	4.9*	0.5	4.5	0.4	0.4
5α-Androstane-3,17-dione	0.6	0.3	0.2	0.4	0.6

Values are expressed as percentage contributions of the listed steroids to the total radioactivity recovered from the TLC plate.

CP = mature canine prostate; HP = human prostate with benign hyperplasia; CE = mature canine epididymis; EA = ethyl acetate; Me/A = methylene chloride:acetone (4:1 v/v).

*cf. text for identification.

Incubation Conditions: 2.43 nmoles, 0.5 μM substrate/0.2 g tissue mince/hr.

Portions of the "Polar Unknown" fractions derived from substrate incubations with unsupplemented hyperplastic canine and NADPH-supplemented benign hyperplastic human prostate preparations (Table 4) were fractionated, utilizing the TLC systems devised by Gustaffsson et al. (13) for the resolution of $C_{19}O_3$-steroids. Most of the "Polar Unknown" radiometabolites had chromatographic mobilities which were consistent with those of androstanetriol epimers. Following CrO_3-oxidation of other portions of the "Polar Unknown" fractions, reaction products were

resolved by TLC with system 2. Autoradiography of the chromatograms visualized 2 radioactive zones isopolar with 6-trione and 7-trione. The lower mobility of the former relative to the latter trione permitted adequate separation of the products. The identity of these derivatives of the "Polar Unknowns" was ascertained by crystallization to constant SA (Table 5). The ratio of 7-trione/6-trione formed was 3.9 for the unsupplemented hyperplastic canine prostate (with a 42% yield of the two androstanetrione epimers), 2.0 for the NADPH-supplemented human prostate

TABLE 5. CRYSTALLIZATION OF CrO_3-OXIDATION PRODUCTS OF THE 5α-ANDROSTANETRIOL RADIOMETABOLITES

ORIGIN OF FRACTION	CRYSTALLIZATION NO.	CRYSTALLIZATION SYSTEM[+]	SPECIFIC ACTIVITY STARTING[*]	SPECIFIC ACTIVITY CR	SPECIFIC ACTIVITY MLQ[‡]
CARRIER: 5α-ANDROSTANE-3,6,17-TRIONE					
HUMAN PROSTATE	1	A/W	367 (14,300)	355	367
	2	M/W		352	356
	3	EA/H		353	349
CANINE PROSTATE	1	A/W	632 (12,000)	395	901
	2	M/W		353	431
	3	A/W		347	358
CARRIER: 5α-ANDROSTANE-3,7,17-TRIONE					
HUMAN PROSTATE	1	A/W	320 (12,500)	302	335
	2	M/W		299	299
	3	EA/H		294	295
CANINE PROSTATE	1	A/W	720 (28,100)	676	751
	2	M/W		670	682
	3	EA/H		654	673

[+]cf. Materials and Methods; [*]DPM in parenthesis; CR = crystals;

[‡]MLQ = redistributed SA of mother liquor (3).

and 2.9 for the NADPH-fortified canine epididymis preparations. The data presented in Table 4 also show that the tissues examined rank in the order of canine epididymis, hyperplastic canine prostate and hyperplastic human prostate in their capacity to hydroxylate 3β-diol and that NADPH-supplementation of the minced preparations enhances the transformations.

DISCUSSION

This paper reports the 3-monoacetylation and 6ξ- and 7ξ-hydroxylation of 3β-diol-^{14}C by canine and human prostatic and canine epididymal preparations.

Ethyl acetate as the extracting solvent was the acetate donor for the acetylation reaction. Accordingly, the use of ethyl acetate as a means of terminating *in vivo* and *in vitro* reactions in which 3-hydroxysteroids are formed or serve as substrate should be avoided. Thus "Unknown 1" radiometabolites, isopolar on TLC with androstenedione, were formed in the canine prostate and epididymis on extraction of the organs with ethyl acetate-acetone after intra-arterial infusion of testosterone-^{14}C (1,2). They are now shown to consist of a mixture of the 3-monoacetates of 3α-diol-^{14}C and 3β-diol-^{14}C of which the prostatic and epididymal 3β-monoacetate constituent *per se* and both epimeric androstanediol hydrolysis products of prostatic origin have been identified by crystallization to constant SA. The *in vivo* and *in vitro* experiments described here provide an indication that the acetylation may be enzymatic.

If the percentage contribution of the 3-monoacetate derivatives to the total radioactivity recovered from the glands is added to that reported for the free epimers formed on infusion (2), the androstanediol share of the prostatic radioactivity is raised from 14.3% to 28.8% and that of the epididymal radioactivity from 25.5% to 45.4%. Preliminary evidence based

on chromatographic separation of the epimeric 17-oxosteroid derivatives and the cited low content of identified 3β-diol-^{14}C 3-monoacetate in the epididymal "Unknown 1" fraction points to the preferential transformation of testosterone-^{14}C to 3α-diol-^{14}C 3-monoacetate in the canine epididymis. The "Unknown 2" radiometabolites (2) yielded products isopolar with isoandrosterone and androsterone on alkaline hydrolysis. These results necessitate a revision of the numerical values for the ratio of 5α-reduced 17β-hydroxysteroids/total 17-oxosteroids (1,2) which are a measure of the relative contributions of the 17β-hydroxysteroid and 17-oxosteroid pathways of testosterone metabolism.

The acetylation reaction accounted for only a minor share of the extensive transformation of 3α-diol and 3β-diol by minced prostate and epididymis. These preparations actively hydroxylate one or both of the 7- and, to a lesser extent, one or both of the 6-positions of 3β-diol-^{14}C Identification is based on CrO_3-oxidation of the unknown polar radiometabolites to 6-trione-^{14}C and 7-trione-^{14}C, structure determination of the latter trione, devoid of the former, by MS after GLC fractionation, and crystallization of both triones to constant SA after two-dimensional separation by TLC. Trione derivatives of polar radiometabolites produced in the infusion experiments (1) had identical chromatographic mobilities.

Evidence for C_{19}-steroid hydroxylation in male accessory reproductive glands and related tissues has hitherto rested on the identification of 2β-hydroxy-4-androstene-3,17-dione as a metabolite of androstenedione-4-^{14}C on incubation with minced normal human prostate (14), of 6β-hydroxy-4-androstene-3,17-dione, in addition to the 2β-hydroxy epimer, in incubates of the same radiosubstrate with minced preparations of pooled hyper-

plastic and carcinomatous human prostate (15), of 7α- and 7β-hydroxy-dehydroepiandrosterone from dehydroepiandrosterone-4-^{14}C by the action of minced normal human epididymis (16), and of 5α-androstane-3β,7α,17β-triol on metabolism of 7-^{3}H-4-^{14}C- and 7β-^{2}H-4-^{14}C-labeled testosterone and 5α-dihydrotestosterone in minced canine perianal glands (17).

Regarding our new findings, the high substrate effectiveness of 3β-diol in the prostatic and epididymal 7-hydroxylation reaction(s) is of particular interest. The enhanced yield of hydroxylation products on NADPH-supplementation points to involvement of mixed-function oxidases. The widely-held assumption that smooth endoplasmic reticulum is the site of mixed-function oxidase activity can account for the presence of hydroxylase activity in the epididymis, the epithelium of which has been shown to contain this organelle in the several species examined (18), but runs counter to the present demonstration of substantial hydroxylase activity in the canine prostate, since the epithelial fine structure of this gland is devoid of smooth endoplasmic reticulum (9). The prostatic hydroxylases may therefore be located in the rough endoplasmic reticulum and/or the Golgi apparatus.

Interest in the metabolic fate of 3α-diol and 3β-diol was stimulated by the identification of both steroids as transformation products of testosterone-4-^{14}C in accessory reproductive organs *in vitro* (9,19-21) and *in vivo* (1,2,22,23), by the substantially higher excretion rate of the urinary androstanediol epimers in men than in women (24-26), and by a considerable difference in the relative magnitude of target-tissue responses to the two androstanediol epimers on systemic as compared with local administration (27-29). *In vivo* radiotestosterone conversion to 3α- and 3β-diol was more extensive in the epididymis than in the

prostate (2,23).

Data presented in this paper reveal the predominance of different oxidative pathways of canine prostate metabolism *in vitro* for the two epimers. Whereas 3β-diol was the preferred substrate for the hydroxylation reactions, the same tissue preparation discriminated greatly in favor of 3α-diol for dehydrogenation to 5α-dihydrotestosterone. Active dehydrogenation of 3α-diol and none of the 3β-epimer had previously been reported by Robel *et al.* (29) for rat ventral prostate in organ culture. These *in vitro* observations may explain the much lower recovery of urinary radioactivity in 3β-diol and total $C_{19}O_2$-steroids from 3β-diol-^3H substrate than in 3α-diol and total $C_{19}O_2$-steroids from administered 3α-diol-^3H after separate intravenous injection of the epimers into two healthy men (30).

The role of the androstanediol epimers in the totality of the androgenic response of testosterone-metabolizing male target tissues continues as a live issue. It remains to be seen whether hydroxylation reactions serve to terminate the hormonal response, accounting *inter alia* for the enhanced potencies of synthetic androgens with 7α-methyl substituents (31), or produce intracellular mediators with distinct biological properties of their own.

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