METABOLISM OF DEHYDROEPIANDROSTERONE[1] AND DEHYDROEPIANDROSTERONE SULFATE IN TISSUE EXTRACTS OF RABBIT OVARIES

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ABSTRACT

The simultaneous incubation of ^{3}H-dehydroepiandrosterone sulfate and ^{14}C-dehydroepiandrosterone by soluble and microsomal extracts of rabbit ovaries was investigated. ^{3}H-dehydroepiandrosterone sulfate was converted to ^{3}H-androstenediol-3-sulfate by both extracts. Only the microsomal extract converted ^{3}H-dehydroepiandrosterone sulfate to ^{3}H-testosterone, ^{3}H-Δ^{4}-androstenedione and ^{3}H-3β-hydroxy,5α-saturated steroids. Both extracts converted ^{14}C-dehydroepiandrosterone to ^{14}C-testosterone, ^{14}C-Δ^{4}-androstenedione and ^{14}C-3β-hydroxy,5α-saturated steroids. Δ^{5}-androstenediol was not detected as a product in the incubation with either the soluble or the microsomal extract.

Dehydroepiandrosterone sulfate (DHA-S) is secreted by the adrenal cortex[2,3] and is present in relatively high concentrations in plasma[4]. The recognition that steroid sulfates can serve as biosynthetic intermediates both *in vivo*[5-7] and *in vitro*[8-11] and the demonstration that ovaries can utilize DHA-S[12] as well as dehydroepiandrosterone (DHA)[13-15] as a precursor for testosterone and androstenedione (Δ^{4}-dione) led us to compare the metabolism of DHA and its sulfate ester in extracts of rabbit ovaries.

EXPERIMENTAL

Materials. 7α-^{3}H-dehydroepiandrosterone (1.62 c/mmole), 4-^{14}C-dehydroepiandrosterone (56 mc/mmole), 4-^{14}C-Δ^{4}-androstenedione (58 mc/mmole) and 7α-^{3}H-dehydroepiandrosterone sulfate, ammonium salt, (1.63 c/mmole) were purchased from New England Nuclear Corporation. ^{3}H-DHA-S was purified by paper chromatography in system PC 1 (described below), ^{3}H-DHA in system Ag-ITLC 1. ^{14}C-Δ^{4}-dione were each judged pure by yielding only one spot on chromatography in system ITLC 3 and Ag-ITLC 1. DHA, testosterone, and $NADPH_2$ were purchased from Sigma Chemical Company. All other non-radioactive steroids employed in this study were obtained from Mann Research Laboratories, Inc.

DHA-S and Δ^{5}-diol-3-S were prepared as the potassium salts as described previously (10). The potassium salt of 5α-3β, 17β-diol-3-S was prepared by sulfurylating 5α-3β-ol,17-one, followed by reduction by sodium borohydride as described for the preparation of Δ^{5}-diol-3-S (10).

Steroids were acetylated in pyridine-acetic anhydride, 2:1 (v/v) overnight at room temperature. The excess reagent was removed under vacuum at 40°C. The residue was dissolved in methylene chloride and washed twice with 1/4 volume water. The solution was dried over magnesium sulfate, filtered, and evaporated. The steroid acetates were crystallized and aliquots chromatographed in either system ITLC 4 or ITLC 5 (see below under chromatography).

Tissue Preparation. Ovaries were obtained from mature rabbits immediately after sacrifice and homogenized in 4 volumes of ice-cold 0.154 M KCl in a teflon-glass Potter-Elvehjem homogenizer. The homogenate was centrifuged at 8000 g for 20 min. The resulting supernate was centrifuged at 100,000 g for 1 hr to obtain the soluble extract and the microsomal pellet. The pellet was rinsed twice with 0.154 M KCl to remove any adhering supernate.

Incubation I. 0.017 μmole of ^{3}H-DHA-S (2.2×10^{7} dpm) and 0.017 μmole of ^{14}C-DHA (2.19×10^{6} dpm) were incubated in duplicate in a medium containing 0.5 mmole potassium phosphate buffer (pH 7.4), 6.7 μmoles $NADPH_2$, and soluble enzyme preparation equivalent to approximately 1.1 g of fresh tissue in a total volume of 4.0 ml. The ^{14}C-DHA was dissolved in two drops of propylene glycol before addition of the medium. Incubation was carried out in a Dubnoff shaker at 37° for 2 hrs.

Incubation II. This procedure was the same as described for I except that microsomal fraction equivalent to approximately 1.1 g of fresh tissue was used.

Incubation III. 0.017 μmole of ^{3}H-DHA (2.04 x 10^{7} dpm) and 0.017 μmole of ^{14}C-Δ^{4}-dione (2.08 x 10^{6} dpm) were incubated in a medium containing 0.3 mmole potassium phosphate buffer (pH 7.4), 3.3 μmoles $NADPH_2$, and microsomal enzyme preparation equivalent to approximately 2.2 g of fresh tissue in a total volume of 3.0 ml. Incubation was carried out in a Dubnoff shaker at 37° for 20 min.

Chromatography Systems. The following chromatography systems were used and will be referred to in the text by number. Paper chromatography (abbreviated PC below) was carried out on Whatman No. 2 paper which had previously been washed with absolute methanol for 72 hrs. Silica gel impregnated glass-fiber sheets (ITLC-SG, Gelman Instrument Company) are referred to as ITLC, or if impregnated with $AgNO_3$ as Ag-ITLC[16].

PC 1 - Isopropyl ether:isobutanol:n-hexane:benzene:NH_4OH:H_2O (75:48:111:186:150:135)[17].

PC 2 - 30% Formamide impregnated paper; cyclohexane:benzene (1:1)[18].

ITLC 1 - $CHCl_3$:Acetone:Acetic Acid (110:35:6); two developments[19].

ITLC 2 - $CHCl_3$[16].

ITLC 3 - Cyclohexane:Ethyl Acetate (7:1)[16].

ITLC 4 - Cyclohexane:Ethyl Acetate (150:8)[16].

ITLC 5 - Cyclohexane:Ethyl Acetate (150:4)[16].

Ag-ITLC 1 - Cyclohexane:Ethyl Acetate (5:1); two developments[16].

Ag-ITLC 2 - Cyclohexane:Ethyl Acetate (7:1); two developments[16].

Ag-ITLC 3 - $CHCl_3$[16].

Extraction and Identification: Following incubation, the steroid sulfates and free steroids were extracted as described previously[20] with the following modification. In incubation I and II the free steroids were extracted with methylene chloride instead of ether.

Application of ITLC systems in conjunction with the P C systems as employed in the present study have been shown to be reliable for identification of the steroids and steroid sulfates to be considered. Detailed description of these systems is presented elsewhere[16,19].

For the identification of steroid sulfates, aliquots of the n-butanol extract were chromatographed in PC 1 for 34 hr, and the resolved products were eluted and chromatographed in ITLC 1[19]. Other PC 1 chromatograms developed in the same manner were subjected to solvolysis[21], followed by elution and sequential chromatography in systems ITLC 3 and Ag ITLC 3.

Isolation of steroid sulfates from Incubation II used the same systems as described for Incubation I; however, the systems were applied in a different sequence. This variation was necessitated because butanol extracts from microsomal fractions contained a tissue component with a mobility in PC 1 which was similar to that of the steroid sulfates under study. This material resulted in considerable tailing of these sulfates and thus in poor resolution. Using ITLC 1 before PC 1 resulted in good resolution of the steroid sulfates and separation from the interfering contaminants. Solvolysis was carried out directly on ITLC[19].

Free steroids from Incubation I were isolated by chromatography of the methylene chloride or ether extract in PC 2, elution of the resulting products, and further chromatography utilizing systems ITLC 2 and 3, and Ag-ITLC 1, 2 and 3. Free steroids from Incubation II and III were also isolated by this procedure and in addition, were acetylated and subjected to chromatography utilizing systems ITLC 4 and 5. Final identification of all isolated products was established by recrystallization with authentic carrier to constant activity.

Per cent recovery of the various products was calculated from the radioactivity found in the final crystals and that added to the incubation mixture. Corrections for losses during isolation and identification were not made.

Detection of Steroids, and Measurement of Radioactivity. Radioactive areas on paper chromatograms, ITLC, and Ag-ITLC were located with a Vanguard Autoscanner 880. Authentic steroid sulfates on paper were detected by the technique of Crepy and Judas[22], free steroids with phosphomolybdic acid reagent[23]. Detection on ITLC and Ag-ITLC of free steroids and steroid sulfates and elution of these compounds from the chromatograms have been described[16,19].

Radioactivity was measured in a Packard Tri-Carb scintillation spectrometer. Simultaneous counts of ^{3}H and ^{14}C were made with efficiencies of approximately 16% and 0% for ^{3}H, and 4% and 30% for ^{14}C in the respective channels. Each sample was counted in 10 ml of toluene containing 4.0 g/l of 2,5-diphenyloxazole (PPO) and 0.1 g/l of 1,4-bis2-(5-phenyloxazolyl)-benzene

(POPOP). Conjugated steroids were first dissolved in 1.0 ml methanol and then 10 ml of the above scintillation solution was added. Appropriate quench corrections were performed using an internal standard.

RESULTS AND DISCUSSION

Table I presents the recrystallization data and per cent recovery of products isolated from the n-butanol extract from Incubation I and II. The results demonstrate the efficient reduction of DHA-S to Δ^5-diol-3-S by both soluble and microsomal fractions of the ovaries. No C^{14} radioactivity was found in any of the steroid sulfates isolated indicating that the conversion occurred by direct reduction. Direct reduction of DHA-S to Δ^5-diol-3-S by a soluble extract of rat testis has been reported in a previous study[10].

The isolation of small amounts of ^{3}H-5α-3β,17β-diol-3-S from the incubation of the soluble fraction with ^{3}H-DHA-S and ^{14}C-DHA (Table I) suggests that there also occurred, without prior hydrolysis of the sulfate ester, either Δ^5 reduction or Δ^{5-4} isomerization followed by Δ^4 reduction. A similar finding was presented by Oertel and Knapstein[24] who reported the isolation of 5α- and 5β-androstan-3β-ol, 17-one from the sulfate fraction in plasma following the i.v. administration of ^{3}H-^{35}S labeled DHA-S.

Although neither 5α-3β,17β-diol-3-S or 5α-3β-ol,17-one-3-S could be detected in the incubation carried out with the microsomal fraction, the isolation of the 5α reduced sulfoconjugated diol from the incubation with the soluble fraction may not reflect the reduction of the Δ^4 or Δ^5 bond of a

Table I

Recrystallization of radioactive compounds isolated from the butanol extract from Incubation I and II.[a]

A. Incubation I (Soluble enzyme fraction)

Compound	^{3}H dpm/mg					
	Initial	C_1	C_2	C_3	M.L.[b]	Per Cent Recovery[c]
Δ^5-diol-3-S	66,012	62,286	63,426	-	66,268	58.0
Δ^5-diol-3-S[d]	17,402	15,085	15,305	15,381	15,329	
5α-3β,17β-diol	-	3,041	3,214	3,421	3,048	0.4
5α-3β,17β-diol-3-S[d]	-	846	856	-	862	
DHA-S[d]	19,827	19,172	18,592	-	19,647	3.6

B. Incubation II (Microsomal enzyme fraction)

Δ^5-diol-3-S	10,055	9,020	8,842	--	9,172	29
Δ^5-diol-3-S[e]	14,862	12,752	12,608	12,402	11,930	
DHA-S	5,958	5,780	5,863	--	4,746	26
DHA-S[e]	12,641	12,715	13,124	--	12,491	

a. See Experimental.
b. Final mother liquor.
c. Derived from ^{3}H-DHA-S.
d. Solvolyzed on paper, eluted, rechromatographed and recrystallized as the free steroid.
e. Solvolyzed on ITLC, eluted, rechromatographed and recrystallized as the free steroid.

3-sulfoconjugated steroid, but may be due to a very efficient reduction of the 17-keto group of 5α-3β-ol,17-one-3-S, which appears as a trace contaminant in ^{3}H-DHA-S. The ^{3}H-DHA-S prior to incubation was chromatographed in system PC 1, which appears to separate this contaminant from DHA-S[17]. It was found, however, that even after careful separation of the contaminating radioactive area from the DHA-S area, there was still a trace of 5α-3βol,17one-3-S which could be detected only after solvolysis of the DHA-S and chromatography on Ag-ITLC 2.

The recrystallization data and per cent recovery of free steroids isolated from the methylene chloride extract in the incubation with the soluble enzyme fraction are presented in Table II. As is seen in this table

Table II

Recrystallization of radioactive compounds isolated from the methylene chloride extract from Incubation I.[a]

Compound	^{14}C dpm/mg					Per cent[c]
	Initial	C_1	C_2	C_3	M.L.[b]	Recovery
Testosterone	13,556	12,799	12,568	-	13,278	42.0
Δ^4-dione	483	536	518	-	508	1.0
5α-3β,17β-diol	-	342	289	294	396	1.0

a. See Experimental
b. Final mother liquor
c. Derived from ^{14}C-DHA

testosterone was the major product of ^{14}C-DHA. Small amounts of Δ^4-dione and 5α-3β,17β-diol were also identified: No Δ^5-diol could be detected. Only ^{14}C radioactivity was found

Table III

Recrystallization or radioactive compounds isolated from methylene chloride extract in Incubation II.[a]

Compound	dpm	Initial	C_1	C_2	C_3	ML[b]
5α-3β,17β-di-acetate	^{14}C/mg	684	563	560	552	586
	^{3}H/mg	847	628	545	536	486
	$^{14}C/^{3}H$	0.8	0.9	1.03	1.03	1.21
Testosterone acetate	^{14}C/mg	21,912	21,442	21,475	--	20,376
	^{3}H/mg	18,908	18,465	18,848	--	17,879
	$^{14}C/^{3}H$	1.16	1.16	1.14	--	1.14
Δ^4-dione	^{14}C/mg	8,008	8,440	8,370	--	8,229
	^{3}H/mg	7,285	7,690	7,191	--	7,042
	$^{14}C/^{3}H$	1.10	1.10	1.16	--	1.17
5α-3-one,17β-acetate	^{14}C/mg	1,117	1,086	1,059	--	1,102
	^{3}H/mg	343	355	321	--	345
	$^{14}C/^{3}H$	3.3	3.1	3.1	--	3.2
5α-3-β-acetate, 17-one	^{14}C/mg	302	265	257	267	--
	^{3}H/mg	177	129	58	65	--
	$^{14}C/^{3}H$	1.71	2.05	4.4	4.1	--
Methylene Chloride Extract	^{14}C			166,000		
	^{3}H			148,000		
	$^{14}C/^{3}H$			1.22		

a. See Experimental.
b. Final mother liquor.

in the free steroids, indicating the absence of sulfatase activity in this soluble tissue fraction.

The products formed in the soluble fraction of the ovaries indicate the presence of Δ^5-Δ^4 isomerase, 3β-dehydrogenase and Δ^4-reductase (5α type) activities, in addition to 17β-dehydrogenase activity. This is in contrast to the enzymatic activities found in the soluble fraction of the rat testis which exhibited only the 17β-dehydrogenase activity[10].

The recrystallization data of free steroids isolated from the methylene chloride extract in incubation II are presented in Table III. The per cent conversion of ^{14}C-DHA and ^{3}H-DHA-S to these products is shown in Table IV.

Table IV

Biosynthesis of androgens and ring A saturated compounds from ^{3}H-DHA-S and ^{14}C-DHA during a 2 hr incubation with microsomal extract (Incubation II).[a]

Compound	Per Cent Conversion from ^{14}C-DHA	Per Cent Conversion from ^{3}H-DHA-S
Testosterone	32.5	2.9
Δ^4-dione	10.5	0.88
5α-3β,17β-diol	0.59	0.042
5α-3-one,17β-ol	2.05	0.058
5α-3β-ol,17-one	0.48	0.014

a. See Experimental

All of the free steroids isolated from the incubation of the microsomal fraction with ^{3}H-DHA-S and ^{14}C-DHA contained some ^{3}H radioactivity (Table III and IV), demonstrating

sulfatase activity in this fraction. The major free steroid produced from both DHA-S and DHA was testosterone. The utilization of DHA-S for androgen synthesis has been reported by Aakvaag et al[12] for the normal canine ovary perfused in vivo. In addition to testosterone a relatively large amount of Δ^4-dione and three ring A saturated C_{19} steroids of the 5α-3β-ol and 5α-3-keto type were isolated. No 3α-ol analogs were detected nor was any Δ^5-diol found. The identification procedure employed in this study has been found to separate the Δ^5-C_{19} steroids from their saturated analogs as well as bringing about resolution of the 3α-ol and 3β-ol analogs and the 5α- and 5β-isomers[16].

Of interest are the $^{14}C/^{3}H$ ratios found in the crystallized free steroids (Table III). Testosterone, Δ^4-dione and 5α-3β, 17β-diol have essentially the same ratio, while this ratio is approximately four times as high in 5α-3β-ol,17-one and approximately 3 times as high in 5α-3-one,17β-ol. This finding suggests that the latter two compounds were not synthesized predominately via Δ^4-dione or testosterone under the experimental conditions used. The $^{14}C/^{3}H$ ratios found could be indicative of direct reduction of the 3β-hydroxy Δ^5 steroid without passing through the 3-keto Δ^4 steroid configuration. To test this possibility, the microsomal fraction was incubated for 20 min with ^{3}H-DHA and ^{14}C-Δ^4-dione. The results from this incubation are presented in Table V. All of the isolated ring A saturated steroids, the testosterone and the Δ^4-dione had similar $^{3}H/^{14}C$ ratios. These results are in agreement with the conventional

Table V

Recrystallization of radioactive compounds isolated from methylene chloride extract in Incubation III.[a]

Compound	dpm	Initial	C_1	C_2	C_3	M.L.[b]	Per Cent Recovery
Testosterone-acetate	^3H/mg	50,373	49,163	49,035	-	48,690	17
	^{14}C/mg	5,202	5,142	5,038	-	5,000	
	$^3H/^{14}C$	9.7	9.6	9.7	-	9.7	
Δ^4-dione	^3H/mg	46,814	45,041	44,306	-	45,563	12
	^{14}C/mg	5,007	4,710	4,710	-	4,700	
	$^3H/^{14}C$	9.3	9.4	9.4	-	9.7	
5α-dione	^3H/mg	4,298	3,910	3,667	3,741	3,721	1.2
	^{14}C/mg	523	473	432	445	463	
	$^3H/^{14}C$	8.2	8.3	8.5	8.4	8.0	
5α-3-one, 17β-acetate	^3H/mg	6,052	5,714	5,519	-	5,683	1.2
	^{14}C/mg	765	781	744	-	744	
	$^3H/^{14}C$	7.9	7.3	7.4	-	7.6	
5α-3β-acetate-17-one	^3H/mg	2,017	1,673	1,549	1,459	1,502	0.6
	^{14}C/mg	213	139	133	144	149	
	$^3H/^{14}C$	9.5	12.0	11.6	10.1	10.1	

a. See Experimental.
b. Final mother liquor.

view that reduction of the Δ^5-3β-ol steroid procedes via the Δ^4-3-keto form. At present the differences found in the two experiments is unexplained.

The isolation of 5α ring A saturated C_{19} steroids in the studies presented here identifies the ovary as another steroidogenic tissue possessing the enzymes necessary for reduction of androgens. In addition to its presence in liver [25], Δ^4-reductases have been reported in adrenal glands of several mammalian species[26], in rat testis[27,28] and recently in human skin[29]. The presence of these enzymes in endocrine organs could function as a control for the amount of androgen available as active hormone or as precursor of estrogens.

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1. The following trivial names and abbreviations have been used.
Dehydroepiandrosterone (DHA), Androst-5-en-17-one; Dehydroepiandrosterone sulfate (DHA-S), Androst-5-en-17-one-3β-yl sulfate; Δ^5-androstenediol (Δ^5-diol), Androst-5-ene-3β,17β-diol; Δ^5-androstenediol-3-S (Δ^5-diol-3-S), Androst-5-ene-17β-ol-3β-yl sulfate; Δ^4-androstenedione (Δ^4-dione), Androst-4-ene-3,17-dione; Testosterone, Androst-4-en-3-one, 17β-ol; 5α-3β,17β-diol, 5α-Androstane-3β,17β-diol; 5α-3β,17β-diol-3-S, 5α-Androstane-17β-ol-3β-yl sulfate; 5α-3β-ol,17-one, 5α-Androstane-3β-ol, 17-one; 5α-3β-ol,17-one-3-S, 5α-Androstan-17-one-3β-yl sulfate; 5α-3-one, 17β-ol, 5α-Androstan-3-one,17β-ol; 5α-dione, 5α-Androstane-3,17-dione.

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