CONVERSION OF DEHYDROEPIANDROSTERONE SULFATE[1] TO ANDROST-5-ENEDIOL-3-SULFATE BY SOLUBLE EXTRACTS OF RAT TESTIS

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Received July 12, 1965

ABSTRACT

^{3}H-Androst-5-ene-17-one-3β-yl sulfate is converted to ^{3}H-androst-5-ene-17β-ol-3β-yl sulfate by soluble extracts of rat testis in the presence of added $NADH_2$ or $NADPH_2$. The conversion was approximately 4 times greater than the conversion of ^{3}H-androst-5-ene-17-one-3β-ol to ^{3}H-androst-5-ene-3β,17β-diol under similar incubation conditions. The same extracts converted ^{3}H-androst-5-ene-17-one-3β-ol to ^{3}H-androst-5-ene-17-one-3β-yl sulfate in the presence of added ATP and Mg^{++}.

INTRODUCTION

In an earlier report from this laboratory[2], it was demonstrated that soluble enzyme preparations from several endocrine tissues, i.e., rat testis and bovine adrenal cortex, ovary, and corpus luteum, sulfurylate estradiol-17β. Recent *in vivo*[3,4,5] and *in vitro*[6,7] studies demonstrating that steroid sulfates can serve as metabolic intermediates led us to investigate whether the above-mentioned preparations also catalyze metabolic inter-conversions of steroid sulfates. In the present investigation it was demonstrated that these preparations sulfurylate dehydroepiandrosterone and that they convert dehydroepiandrosterone sulfate to androst-5-enediol-3-sulfate. Possible relations of these observations to the *in vivo* conversion of dehydroepi-androsterone and its sulfate to testosterone are discussed.

EXPERIMENTAL

Materials

7α-^{3}H-Dehydroepiandrosterone (DHA) (1.15 c/mM) and the ammonium salt of 7α-^{3}H-Dehydroepiandrosterone sulfate (DHA-S) (1.7 c/mM) were purchased from New England Nuclear Corporation. DHA-S was purified by paper chromatography in 0.4 M $NaHCO_3$[2]. The purity of DHA was checked by paper chromatography in hexane-benzene/formamide. Androst-5-ene-3β,17β-diol, dehydroepiandrosterone, $NADH_2$ and $NADPH_2$ were obtained from Sigma Chemical Company.

Preparation of carrier sulfates

DHA was sulfurylated by a modification of the method described by Morren[8]. Pyridine (4.2 ml) was added dropwise with shaking to 1.5 ml of chlorosulfonic acid in 60 ml of CCl_4. Three grams of DHA dissolved in a minimum amount of chloroform was added dropwise to the reaction mixture. The mixture was allowed to stand for 30 minutes in the cold, and then was heated on a steam bath for 30 minutes. After cooling the solid material was collected and stirred with a mixture of 25 ml of water and 13.5 ml of concentrated ammonium hydroxide. Pyridine was removed by ether extraction and residual ether was removed by evaporation. Boiling water (65 ml) was added. The solution was heated to dissolve solid material and was then filtered and allowed to cool. The crystals were collected and dried. They were recrystallized from water.

The ammonium salt of androstenediol-3-sulfate was prepared by reducing DHA-S. Two g of the ammonium salt of DHA-S was dissolved in 50 ml methyl alcohol and the solution was cooled in ice. Sodium borohydride (200 mg) was added. The solution was then warmed to room temperature and allowed to stand overnight. Acetone (8 ml) was added to decompose the excess sodium borohydride. After addition of 2.4 ml of water the solution was placed in the freezer. The sodium metaborate which separated was filtered off, and the solution was evaporated to dryness. The residue was recrystallized from a mixture of methyl alcohol and ether.

The ammonium salt of androstenediol-3-sulfate-17-acetate was prepared from the ammonium salt of androstenediol-3-sulfate by dissolving the latter in pyridine-acetic anhydride, 2:1 (v/v), and allowing the solution to stand overnight at room temperature. The excess reagent was removed under vacuum with slight warming and the residue recrystallized from a mixture of methanol and ether.

Melting points and infrared spectra of the DHA-S and androstenediol-S preparations agreed well with those previously published[5]. Each compound migrated as a single component on paper chromatography in system SL-1 and, after solvolysis, in systems Z-1, Z-2 and Z-3 (see Results).

Incubation and extraction

Testes were obtained from Holtzman rats. Soluble extracts of the tissue were prepared, lyophylized, and stored at -10° as described earlier[9].

Seven μc ^{3}H-DHA-S (0.0226 μmoles) or 6.25 μc ^{3}H-DHA (0.0171 μmoles) was incubated in a rotary agitator for 2 hrs at 37° in one ml of 0.2 M potassium phosphate buffer (pH 7.4) containing lyophylized testis extract equivalent to 10.5 mg of protein and 10 mg of $NADH_2$ or $NADPH_2$. The DHA was first dissolved in 0.03 ml of absolute ethanol before addition to the buffer solution. After incubation, the steroid sulfates and free steroids were extracted as described previously[2].

Detection of steroids and measurement of radioactivity

Radioactive compounds on chromatograms were localized with a Vanguard Autoscanner 880; authentic steroid sulfates were detected by the technique of Crepy and Judas[10], free steroids with phosphomolybdic acid reagent[11]. Radioactivity was measured in a Packard Tri-Carb scintillation spectrometer. The scintillation solution consisted of 0.3% 2,5-diphenyloxazole (PPO) and 0.01% 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) in toluene (w/v).

Relative amounts of the radioactive products on chromatograms were determined by cutting out the specific area, submerging it in scintillation solution, and counting.

The specific activities of ^{3}H-DHA-S and of ^{3}H-androstenediol-3-sulfate-17-acetate was determined by dissolving weighed amounts of the crystals in a known volume of methanol and counting duplicate 0.2 ml aliquots in 7 ml of scintillation solution. Radioactivity is reported without correction for quenching.

RESULTS

Identification--a. Steroid sulfates--The aqueous extracts from the DHA-S incubations containing the steroid sulfates were chromatographed in duplicate in the Schneider-Lewbart system No. 1 (SL-1)[12],

along with authentic steroid sulfates. Only one radioactive area was observed. Since this system did not separate DHA-S from androstenediol-3-sulfate, the radioactive area from one chromatogram was eluted with methanol, divided into two samples, and evaporated to dryness. To one of the samples, 40 µg of authentic androstenediol-3-sulfate was added. Both samples were then subjected to acetylation (see Experimental). The resulting products were dissolved in methanol and rechromatographed in SL-1. Two radioactive areas were observed, one with the mobility of DHA-S, the other with the mobility of androstenediol-3-sulfate-17-acetate. After localizing the radioactive areas, the chromatogram derived from the sample to which carrier androstenediol-3-sulfate had been added before acetylation was treated with the Crepy reagent to determine that all of the added carrier had been acetylated and that the radioactive product migrated with authentic androstenediol-3-sulfate-17-acetate.

The other chromatogram was used to determine the relative amounts of the two compounds detected after acetylation. Following counting, in scintillation fluid the radioactive compound which migrated with the authentic androstenediol-3-sulfate-17-acetate was recovered from the paper by elution with methanol and evaporation to dryness. The residue was dissolved in water and filtered to remove material derived from the scin-

TABLE 1

PROOF OF RADIOCHEMICAL PURITY OF THE ACETATE DERIVATIVE OF ENZYMICALLY-FORMED ^{3}H-ANDROSTENEDIOL-3-SULFATE

Crystallization[a]	Weight (mg.)	Specific Activity (c.p.m./mg.)
After Addition of Carrier Androstenediol-3-sulfate-17-acetate	30.1	11.2×10^{2}
First Crystallization	25.2	11.3
Mother Liquor	4.6	11.4
Second Crystallization	15.7	11.8
Mother Liquor	7.7	12.1
Third Crystallization	13.4	11.7
Mother Liquor	1.4	11.6

[a]Crystallization of a representative sample from methanol-ether

tillation solution as described by Wallace and Silberman[13]. Carrier androstenediol-3-sulfate-17-acetate (30.1 mg) was added to this aqueous solution and evaporated to dryness. The residue was recrystallized three times from methanol-ether without significantly altering the specific activity (Table 1).

Additional evidence that the product formed from DHA-S was androstenediol-3-sulfate was obtained by subjecting the other SL-1 chromatogram to solvolysis for

180 minutes[12]. The solvolyzed products were eluted with methanol, the methanol evaporated, and the free steroids extracted three times from water into methylene chloride. The methylene chloride was evaporated and the products applied to paper along with authentic DHA and androstenediol for chromatography (Table 2) in two of the following systems: formamide as stationary phase and hexane-benzene, 1:1 (v/v), as mobile phase (Z-1 system); formamide as stationary phase and benzene as mobile phase (Z-2 system)[14]; triethylene glycol as stationary phase and toluene as mobile phase (Z-3 system)[15]. Two radioactive areas were observed, one migrating with DHA and the other with androstenediol. The two radioactive areas were cut out and counted to determine the relative amounts of the two compounds (Table 2).

The ether extracts from the DHA-S incubations were also chromatographed in the above systems. No free steroid was detected at the most sensitive setting of the Vanguard scanner[16], indicating that no cleavage of DHA-S to the free steroid had occurred during the incubation and isolation procedures.

b. Free steroids--The dried ether extracts containing the free steroids from the DHA incubation were chromatographed (Table 3) on methanol-washed Whatman No. 1 paper along with authentic steroids in two of the three systems as described for the solvolyzed steroid sulfates. In each of the three systems, only two radioactive areas were

TABLE 2

CONVERSION OF ^{3}H-DHA-S TO ^{3}H-ANDROSTENEDIOL-3-SULFATE BY A SOLUBLE EXTRACT OF RAT TESTES[a]

Experiment No.	Added Cofactor	Radioactivity Measured After	Chromatography System	Per Cent Conversion[b]
I	$NADPH_2$ (10 mg)	solvolysis	Z-1	69
		solvolysis	Z-2	69
II	$NADPH_2$ (10 mg)	solvolysis	Z-1	74
		solvolysis	Z-3	75
		acetylation	SL-1	72
III	$NADH_2$ (10 mg)	solvolysis	Z-1	52
		solvolysis	Z-2	53
IV	$NADH_2$ (20 mg)	solvolysis	Z-1	59
		solvolysis	Z-2	48
		acetylation	SL-1	50

[a]Seven µc ^{3}H-DHA-S (0.0226 µmoles) was incubated in a rotary agitator for 2 hrs at 37° in one ml of 0.2 M potassium phosphate buffer (pH 7.4) containing lyophylized testis extract equivalent to 10.5 mg of protein and the indicated amount of $NADH_2$ and $NADPH_2$.

[b]Per cent conversion = (counts/min of ^{3}H-androstenediol-3-sulfate) ÷ (counts/min of ^{3}H-DHA-S) + (counts/min of ^{3}H-androstenediol-3-sulfate) x 100.

observed, one migrating with DHA and the other with androstenediol. These areas were cut out and counted in scintillation vials to obtain the relative amounts of the two compounds (Table 3). The aqueous extracts from the DHA incubations were chromatographed in the SL-1 system and scanned for radioactivity. No steroid sulfates

were detected at the most sensitive setting of the Vanguard scanner[16].

TABLE 3

CONVERSION OF ^{3}H-DHA TO ^{3}H-ANDROSTENEDIOL BY A SOLUBLE EXTRACT OF RAT TESTES[a]

Experiment	Added Cofactor	Chromatography System	Per Cent Conversion[b]
V	$NADPH_2$ (10 mg) + $NADH_2$ (10 mg)	Z-1 Z-2	14 18
VI	$NADPH_2$ (10 mg)	Z-1 Z-3	18 21
VII	$NADH_2$ (10 mg)	Z-1 Z-3	12 13
VIII	$NADH_2$ (20 mg)	Z-1 Z-2	11 10

[a]Tritiated ^{3}H-DHA (6.25 μc; 0.0171 μmoles) was incubated in a rotary agitator for 2 hrs at 37° in one ml of 0.2 M potassium phosphate buffer (pH 7 4) containing lyophylized testis extract equivalent to 10.5 mg of protein and the indicated amount of $NADH_2$ and $NADPH_2$.

[b]Per cent conversion = (counts/min of ^{3}H-androstenediol ÷ (counts/min of ^{3}H-DHA) + (counts/min of ^{3}H-androstenediol) x 100

Extent of conversion--Between 93.7% and 99.9% of the incubated radioactivity was recovered in the extracts. Since the substrate and one product were the only detectable radioactive compounds on chromatograms of

these extracts, it seemed appropriate to express the extent of conversion to the product as a per cent of the sum of the counts/min of extracted product plus counts/min of extracted substrate. Per cent conversion

TABLE 4

CRYSTALLIZATION OF ENZYMICALLY-FORMED ^{3}H-DEHYDROEPIANDROSTERONE-SULFATE TO CONSTANT SPECIFIC ACTIVITY

Crystallization[a]	Weight (mg.)	Specific Activity (c.p.m./mg.)
After Addition of Carrier Dehydroepiandrosterone-Sulfate	18.7	6.0×10^2
First Crystallization	13.8	5.6
Mother Liquor	4.3	8.6
Second Crystallization	8.5	4.6
Mother Liquor	4.1	5.3
Third Crystallization	4.8	4.7
Mother Liquor	3.0	4.5

[a]Crystallized from methanol-ether

of DHA-S to androstenediol-3-sulfate and of DHA to androstenediol are presented in Tables 2 and 3 respectively. The data demonstrate that the conversion of DHA-S to androstenediol-3-sulfate was approximately four times greater than the conversion of DHA to androstenediol.

A somewhat greater degree of conversion was obtained with $NADPH_2$ than with $NADH_2$ with both substrates.

Sulfurylation of DHA--To determine whether the testicular extracts could sulfurylate DHA, 12.5 µc DHA (0.0342 µmoles) was incubated in phosphate buffer containing testicular extract equivalent to 10.5 mg protein plus ATP and Mg^{++} as described earlier for estradiol-17β[2]. The aqueous extract obtained by the extraction procedure[2] was chromatographed in the SL-1 system. A radioactive area migrating with reference DHA-S was observed. This product was eluted, added to carrier DHA-S and recrystallized to constant specific activity (Table 4). The amount of DHA sulfurylated represented less than 1% of the incubated DHA. Total recovery of the radioactivity added as DHA in two incubation mixtures was 99.9% and 96.6%.

DISCUSSION

Direct interconversion of DHA-S and androstenediol-3-sulfate has been observed *in vivo*. Baulieu *et al.*[3] demonstrated the direct conversion of androstenediol-3-sulfate to DHA-S in humans and reported preliminary observations suggesting that the reverse reaction takes place. Roberts *et al.*[5] also presented evidence for the direct reduction in humans. The present study demonstrates this conversion in a soluble enzyme preparation from rat testis.

That the interconversion as reported in this study

occurred by direct reduction, rather than by intermediate hydrolysis, reduction, and sulfurylation is supported by the observations that: a) the extent of conversion of DHA-S to androstenediol-3-sulfate in 2 hours was 4 times as great as the conversion of DHA to androstenediol, b) no radioactive free steroid was detected after incubation of DHA-S, and c) no radioactive conjugated steroid was detected after incubation of DHA in the absence of added ATP and Mg^{++}. An indirect interconversion would not be expected from observations *in vitro* with other tissues showing that: a) sulfatase activity is largely restricted to the microsome fraction[17] and, when present, is strongly inhibited by inorganic phosphate[18,19], and b) sulfurylation requires added ATP and Mg^{++}[20,21].

The present study identifies rat testis as one tissue possessing enzymes capable of catalyzing the reduction of DHA-S to androstenediol-3-sulfate. It is probable that this activity is shared by other tissues that have 17-dehydrogenase activity. It occurs in tissues other than testis since it has been demonstrated *in vivo* in human females[3,5]. The reaction has also been reported to occur in rabbit liver slices[22]. We have found in preliminary studies that soluble extracts of bovine ovary also catalyze the reduction but were less active than the rat testis extracts.

Present speculation concerning the role of the

reduction of DHA to androstenediol in steroid metabolism is concerned with the possibility that this may be the first reaction in the conversion of DHA to testosterone. The utilization of DHA in the biosynthesis of testosterone has been established *in vivo*[23,24] and *in vitro*[25]. Recent reports by several authors [24,26,27,28] provide evidence that a major pathway exists for the synthesis of testosterone from DHA that does not involve androstenedione as an obligatory intermediate, and these authors suggest androstenediol may be the intermediate. This suggestion is supported by reports of the conversion of androstenediol to testosterone by bacterial preparations[29], by rat testicular suspensions[30], and by homogenates of human placenta and adrenocortical tumor[31]. Also, Klempien *et al.*[32], using isolated dog liver, identified androstenediol as a major intermediate in the conversion of DHA to testosterone. In a more recent study, Rosner *et al.*[25] reported the isolation of androstenediol from rabbit testis homogenates that had been incubated with DHA and the conversion of the enzymatically-synthesized androstenediol to testosterone by the same homogenates.

The conversion of DHA-S into testosterone has been demonstrated by Aakvag *et al.*[33] in perfused canine testis and ovary. However, the reaction sequence in this conversion was not demonstrated. They were unable to detect any free DHA in the spermatic vein after infusion

of DHA-S into the spermatic artery, suggesting that DHA-S may be reduced to androstenediol-3-sulfate prior to the cleavage of the sulfate ester to the free steroid. The finding in the present study that DHA-S was converted to androstenediol-3-sulfate by rat testis extracts and that this conversion was much more efficient than the conversion of DHA to androstenediol provides support for this suggestion.

ACKNOWLEDGEMENTS

This investigation was supported in part by two research grants, AM-02294-07 from the United States Public Health Service and MMPP No. 299 from the Michigan Memorial Phoenix Project, The University of Michigan.

The authors wish to thank Mr. John P. Francis for the preparation and characterization of the reference steroid sulfates.

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1. The following trivial names and abbreviations have been used: dehydroepiandrosterone, DHA = 3β-hydroxyandrost-5-ene-17-one; dehydroepiandrosterone sulfate, DHA-S = androst-5-ene-17-one-3β-yl sulfate; androstenediol = androst-5-ene-3β,17β-diol; androst-5-enediol-3-sulfate, androstenediol-3-sulfate = 17β-hydroxyandrost-5-ene-3β-yl sulfate; androstenediol-3-sulfate-17-acetate = androst-5-ene-3β-yl-sulfate-17β-yl-acetate.

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