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IDENTIFICATION OF 5α-ANDROSTANE-3β,17β-DIOL AND 3β-HYDROXY-5α-ANDROSTAN-17-ONE SULFATES AS QUANTITATIVELY SIGNIFICANT SECRETORY PRODUCTS OF PORCINE LEYDIG CELLS AND THEIR PRESENCE IN TESTICULAR VENOUS BLOOD

J. I. RAESIDE,* R. L. RENAUD and D. E. MARSHALL
Department of Biomedical Sciences, University of Guelph, Guelph, Ontario, Canada N1G 2W1

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Summary—By means of high performance liquid chromatography and gas chromatography–mass spectrometry it has been found that 5α-androstane-3β,17β-diol sulfate and 3β-hydroxy-5α-androstan-17-one sulfate (epiandrosterone) are major secretory steroids of the mature boar testes. These same compounds were similarly identified in culture media when porcine Leydig cells were incubated with androstenedione as substrate. In addition, they were seen as the principal secretory products when [^{3}H]androstenedione and [^{3}H]testosterone were used as substrates; and their presence was greatly reduced by an inhibitor of 5α-reductase (*N,N*-diethyl,4-methyl-3-oxo-4-aza-5α-androstane-17β-carboxamide). Greater quantities of 5α-androstanediol than epiandrosterone were noted in all instances. These findings provide further evidence of the versatile activity of the boar testes in steroidogenesis.

INTRODUCTION

A feature of steroid metabolism in the testes of the mature domestic pig is the active biosynthesis of steroid sulfates [1–3], and the secretion of some of these compounds, such as dehydroepiandrosterone sulfate (DHAS) [4], Δ^5 androstenediol sulfate (Δ^5AS) [5], and estrogen sulfates [6–8]. Studies of isolated porcine Leydig cells have shown these to be the site in which steroid sulfation occurs [3, 9]. Partial characterization of steroid sulfotransferase activities in these cells has been reported recently [10].

In the course of further study on factors regulating steroid metabolism in the Leydig cells of the boar testis, the use of radioactive steroids gave rise to the consistent appearance of prominent products, not identified by us heretofore, as secreted sulfoconjugated steroids. The isolation and characterization of both 5α-androstane-3β,17β-diol, and 3β-hydroxy-5α-androstan-17-one as principal steroids in the solvolyzable fraction of media extracts from the incubation of porcine Leydig cells, is described in this report. But 5α-reduced steroids were also identified in testicular venous blood and thus constitute a major form of steroid production by the boar testes.

*To whom correspondence should be addressed.

EXPERIMENTAL

Steroidal substrates

[1,2,6,7-^{3}H]Androstenedione (sp. act. = 101 Ci/mmol) and 5α-[1,2-^{3}H]androstane-3β,17β-diol (sp. act. = 45 Ci/mmol) were purchased from Amersham Canada Ltd., Oakville, Ontario. [1,2,6,7-^{3}H]testosterone (sp. act. = 89.1 Ci/mmol) was supplied by NEN Dupont (Canada) Markham, Ontario.

The following non-radioactive steroids were obtained from Steraloids Inc (Wilton, NH, U.S.A.): androstenedione (A), 5α-androstane-3β,17β-diol (3β-A), 3β-hydroxy-5α-androstan-17-one (epiandrosterone), testosterone (T), 4-hydroxyandrostenedione (4-OH A), and 17β-hydroxy-1,4-androstadien-3-one (1,4 T). The sample of 5α-reductase inhibitor *N,N*-diethyl-4-methyl-3-oxo-4-aza-androstane-17β-carboxamide (4-Aza) was kindly supplied by Dr G. H. Rasmusson, Merck Sharp & Dohme Research Lab (Rahway, NJ, U.S.A.).

Other materials

Percoll was purchased from Pharmacia (Canada) Ltd., Dorval, Quebec. Solvents were glass-distilled, reagent or HPLC grade from Caledon Laboratories, Ltd. (Georgetown, Ontario, Canada). All other chemicals were of analytical grade from Sigma Chemical Co.

(St Louis, MO, U.S.A.) or Fisher Scientific (Toronto, Ontario).

Preparation and incubation of Leydig cells

Preparation and purification of porcine Leydig cells were performed as described earlier [11]. Collagenase dispersed and Percoll-purified Leydig cells from mature male pigs were incubated ($10–20 \times 10^6$ cells) for 15–180 min at 32–34°C under 95% air:5% CO_2 in 3–10 ml of medium TC199 in 25 ml-capacity Erlenmeyer flasks in a Dubnoff shaking waterbath. Non-radioactive A and T were added as substrates in ethanol to give a final concentration of 6×10^{-6} M and <0.1% of ethanol in the medium. The same substrates as radiolabelled compounds (5×10^6 cpm of [^{3}H]steroid/flask) were evaporated to dryness under nitrogen before the addition of medium and cells. When inhibitors (6×10^{-6} M) were used the cells were exposed to them for 15 min before adding radiolabelled substrate. At the end of the incubation period the cells were removed by centrifugation or by processing the media through an extraction cartridge immediately. When stored the media samples were kept at −20°C until analysed. All experiments were repeated twice with cells from two other animals.

Analytical procedures

Steroids in samples of media from cell incubations were recovered for separation on high performance liquid chromatography (HPLC) by solid-phase extraction with Sep-Pak C_{18} cartridges (Waters Scientific, Mississauga, Ontario). Unconjugated and conjugated steroids were eluted from the primed cartridges with 5 ml diethyl ether and 5 ml methanol, successively. After evaporating off the ether under nitrogen the residue (unconjugated fraction) was dissolved in 200 μl acetonitrile. In the case of the conjugated fraction (methanol), it was taken to dryness under nitrogen and redissolved in 0.5 ml methanol. For solvolysis, 5 ml of an acidified solution of ethyl acetate (10 drops of 6 M H_2SO_4 in 100 ml of EtOAc) was added and the mixture was kept in a glass-stoppered test tube overnight at 45°C. Then the ethyl acetate solution was washed once with 1 ml $NaHCO_3$ and once with 1 ml double-distilled water before evaporating under nitrogen. The dried residue was dissolved in 200 μl acetonitrile for HPLC. Separation of steroids was made by injecting 10–50 μl of the sample onto a radial compression C_{18}, 4 μm column (Waters), attached with a guard column in a Waters HPLC system, using a solvent system of acetonitrile–water (37:63, v/v) at a flow rate of 1 ml/min and a u.v. detector set at 254 nm. Fractions (0.5 ml) were collected automatically (Pharmacia, LKB-RediFrac); where radiolabel was used, aliquots were taken for scintillation counting (5 ml ACS, Amersham). In the other experiments, fractions were pooled from appropriate peaks, or retention times, and solvents were evaporated. Further characterization was done by capillary column gas chromatography–mass spectrometry (GC–MS) on underivatized products isolated from HPLC of the steroid fraction. GC–MS was carried out on a HP 5890 series gas chromatograph interfaced to a Finnigan Mat Ion Trap. GC was performed using a RTX_5 (Restek) column (30 m × 0.32 mm id, 0.25 μ coating) with helium as carrier gas, injection temp 100°C. Mass spectra were generated over 100–450 scan.

RESULTS

The profiles on HPLC for the metabolites of [^{3}H]A and [^{3}H]T showed marked differences, in each case, between the unconjugated steroids and the solvolyzable steroids from the incubation media (Fig. 1). Amongst several prominent peaks in the conjugated fraction, the one at the retention time of just under 26 min was dominant for both steroid substrates. Most of the unconjugated metabolites were present as polar products and little evidence of unmetabolized radioactive substrate was seen. Retention times for T and A were 23.3 and 32.2 min, respectively. In the sulfoconjugated fractions the qualitative profiles obtained from both substrates were very similar, more radioactive products occurred as less polar peaks with those at 25.5 and 40.5 min predominating.

These same peaks (25.5 and 40.5 min) were even more pronounced when an attempt was made to inhibit estrogen formation by adding the aromatase inhibitor, 4-OH A, before incubation of cells with [^{3}H]A (Fig. 2). Practically no other peaks were seen in the sulfoconjugated fraction. It was noted also that greater amounts of radioactivity remained in the unconjugated fraction for these two peaks.

In the course of further study, a striking pattern for the sulfoconjugated products of [^{3}H]A was observed when 1,4 T was included as a competitive substrate in the medium (Fig. 3). The peaks at 25.5 and 40.5 min almost stood

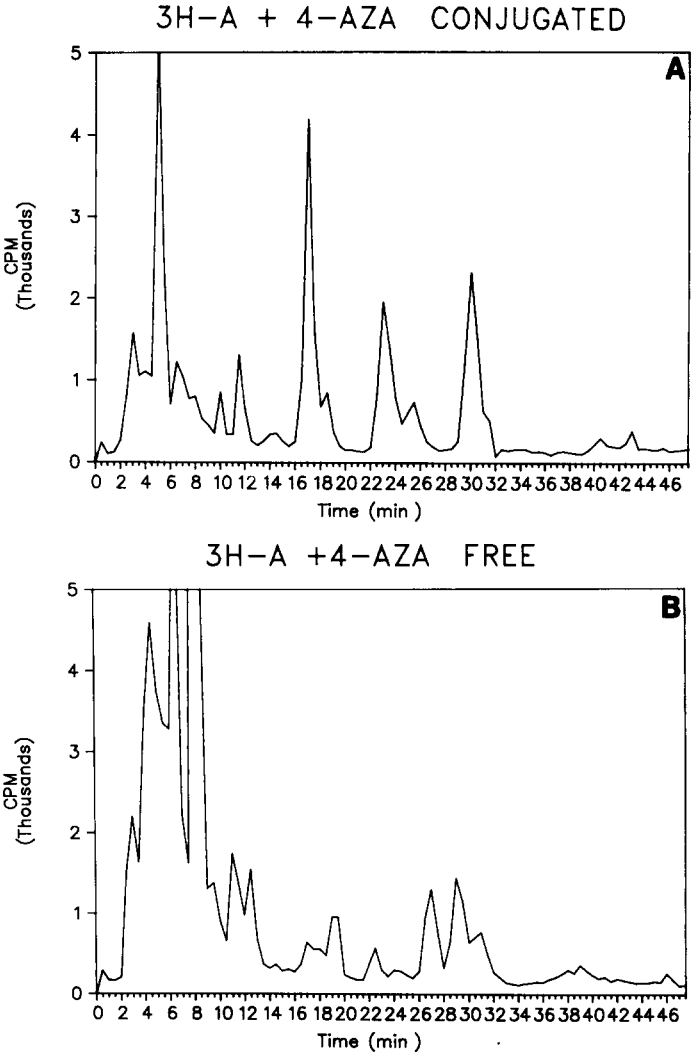

Fig. 4. HPLC distribution of radioactivity after incubation of porcine Leydig cells with [^{3}H]A in the presence the 5α-reductase inhibitor (4-Aza, 6×10^{-6} M). Note almost complete suppression of peak at 25.5 min in the conjugate fraction A; compare with Fig. 1(A).

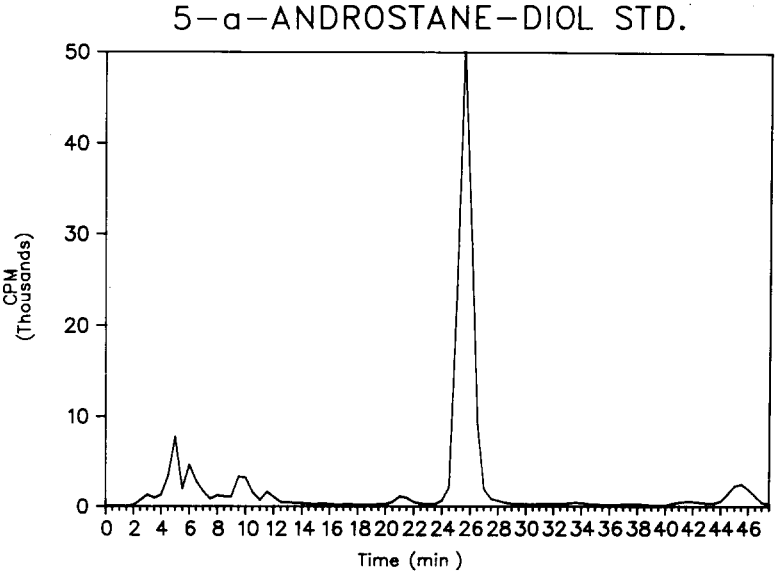

Fig. 5. HPLC of authentic standard of [^{3}H]3β-A.

amounts than 5α-androstanediol, was likewise confirmed for both testicular vein blood (Fig. 6) and Leydig cell incubation media (data not shown).

DISCUSSION

In this study it has been demonstrated that a significant proportion of the metabolites of both T and A are secreted in a sulfoconjugated form by porcine Leydig cells. These results are not surprising in view of the known secretion of steroid sulfates by boar and human testes [4–8, 12], and later evidence for steroid sulfation by porcine Leydig cells [9]. In the latter study, a broad, symmetrical peak was noted for the distribution of radioactivity after partition column chromatography on Celite of the solvolyzed neutral fractions from an incubation with [^{3}H]A. The retention time of the peak suggested the presence of androstanediol-like material. A further basis for this assumption lay in the relatively large amounts of the various epimeric forms of androstanediol isolated previously [13] from the mono-sulfate fraction of boar testicular extracts. The androstanediols were found to occupy an important position quantitatively among boar testicular steroids.

Higher resolution by HPLC in the present study revealed a prominent peak in the [^{3}H]-labeled, solvolyzed fraction which had the same retention time (25.5 min) as that for an authentic standard of [^{3}H]3β-A. Furthermore,

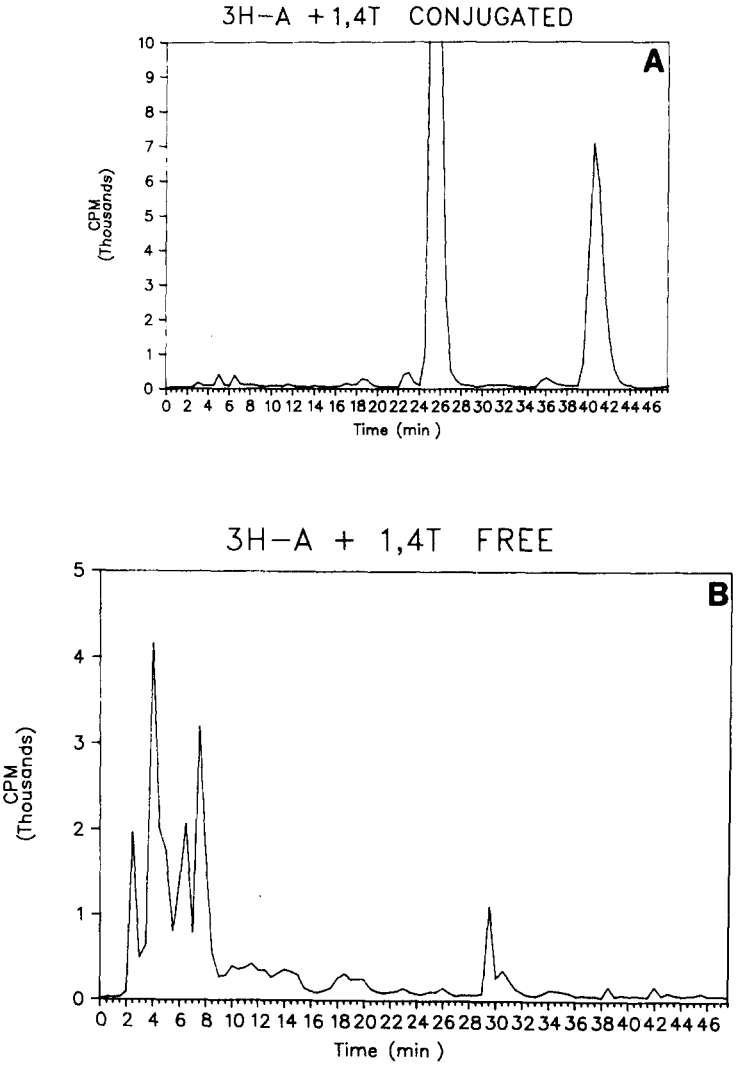

Fig. 3. HPLC distribution of radioactivity after incubation of porcine Leydig cells with [^{3}H]A in the presence of 1,4-androstadien-17-ol-3-one (1,4 T; 6×10^{-6} M). Note prominent peaks at 25.5 and 40.5 min in the conjugated (A) fraction.

alone, with the first being much greater, as noted above for both [^{3}H]A and [^{3}H]T in Fig. 1. These peaks had not been detected previously when non-radioactive A had been used as substrate and chromatographic separations scanned at 254 nm. Loss of the chromophore (Δ^4 3-oxo) in formation of saturated compounds was suspected. For this reason, an inhibitor of 5α-reductase (4-Aza) was added to the medium. The resulting HPLC profile in Fig. 4 shows an almost complete elimination of the peaks at 25.5 and 40.5 min, suggesting that the peaks of interest in the incubations with [^{3}H]A alone may represent 5α-reduced products.

Since a standard of [^{3}H] 3β-A was shown to have a retention time of about 25.5 min (Fig. 5), we collected material from the same position at the HPLC separation of products from the incubation of Leydig cells with non-radioactive A. The peaks at 25.5 min from several runs were combined, and subsequently identified by GC–MS as 3β-A (Fig. 6). Using this technique, it was found that blood from the testicular vein of a mature boar also contained 3β-A, in the solvolyzable fraction, identified by GC–MS (data not shown). The less polar metabolite with a retention time of 40.5 min was shown to be epiandrosterone by GC–MS. Its presence, although in lesser

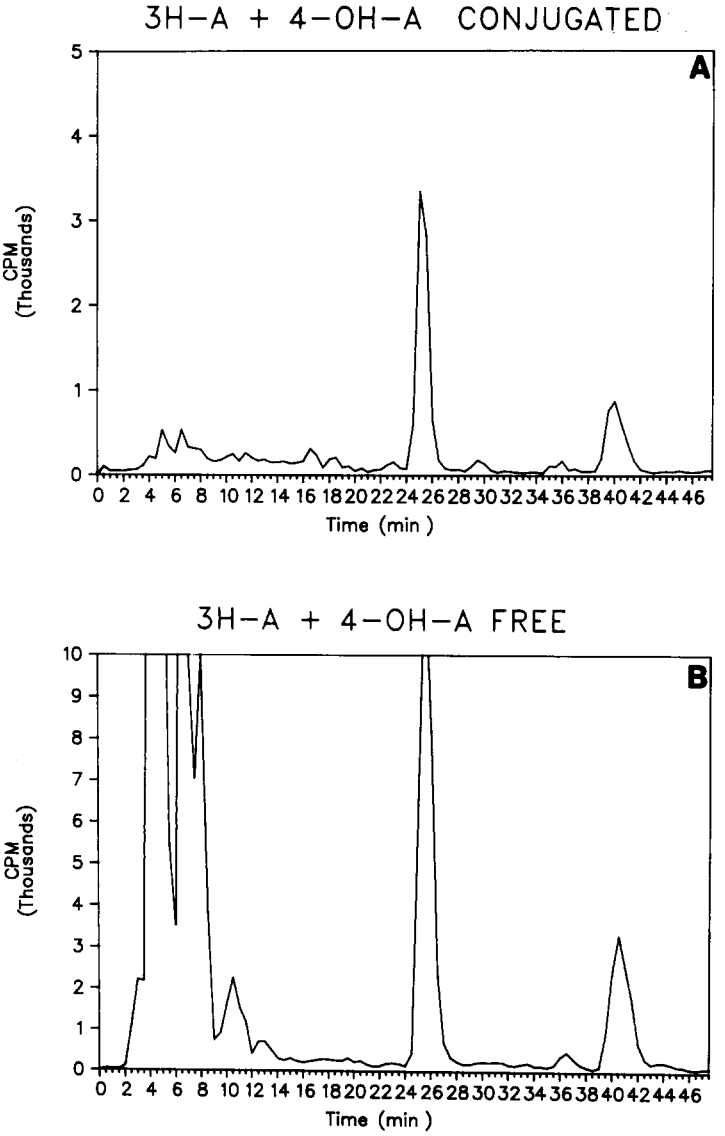

Fig. 2. HPLC distribution of radioactivity after incubation of porcine Leydig cells with [^{3}H]A in the presence of 4-OH A (6×10^{-6} M). Note peak at 25.5 min in conjugated (A) and unconjugated (B) fractions.

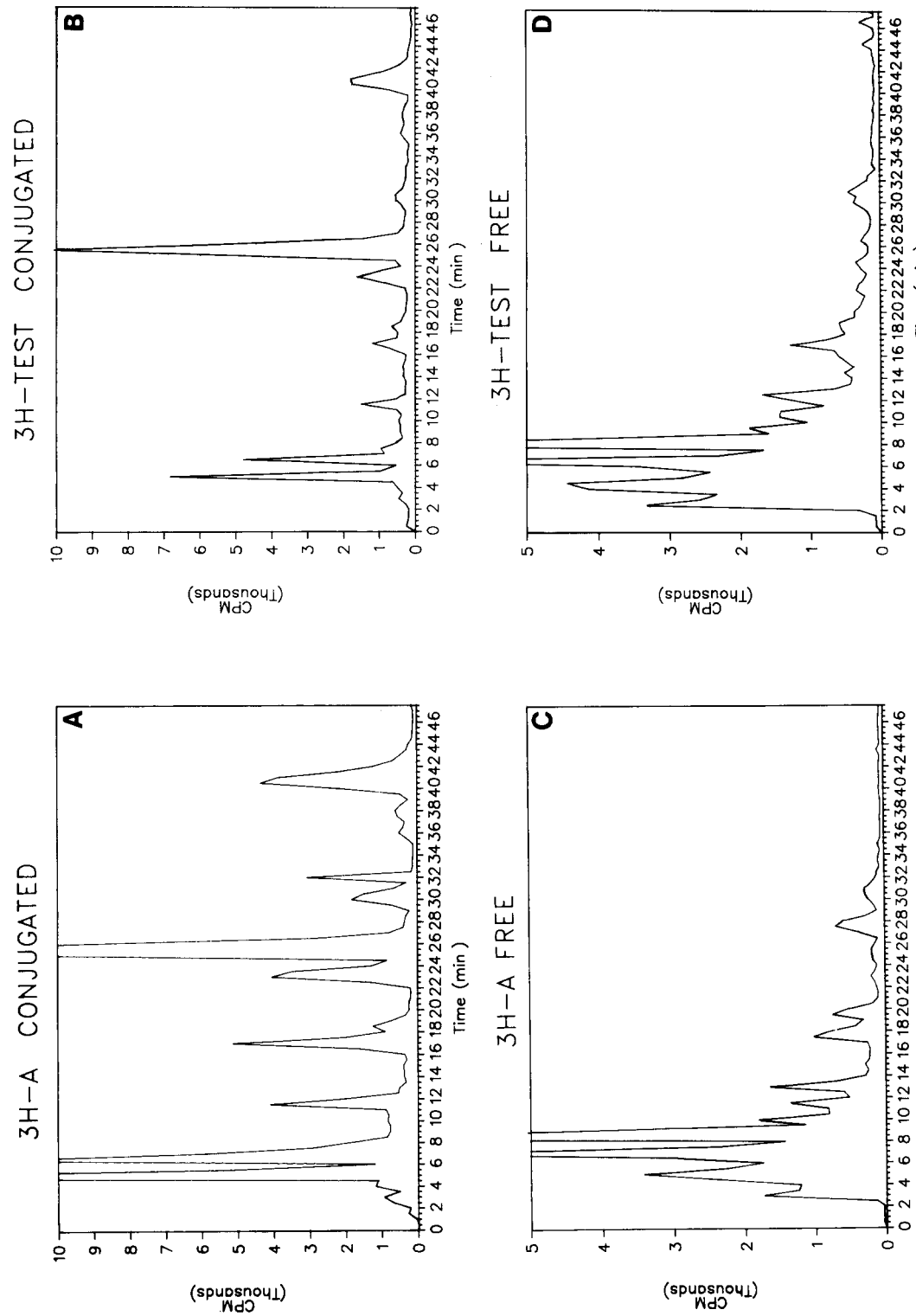

Fig. 1. HPLC distribution of radioactivity after incubation of porcine Leydig cells with [^{3}H]A or [^{3}H]T in the medium. Conjugated fractions represent material run after solvolysis of the more polar extracts of the media (see Experimental). Note prominent peak at 25.5 min in conjugated (A,B) lacking in unconjugated fractions (C,D).

in spite of an absence of $\Delta^4$3-oxo-conjugation it was possible to isolate 3β-A in extracts from incubation media with non-radioactive A as substrate, as well as from testicular vein blood,

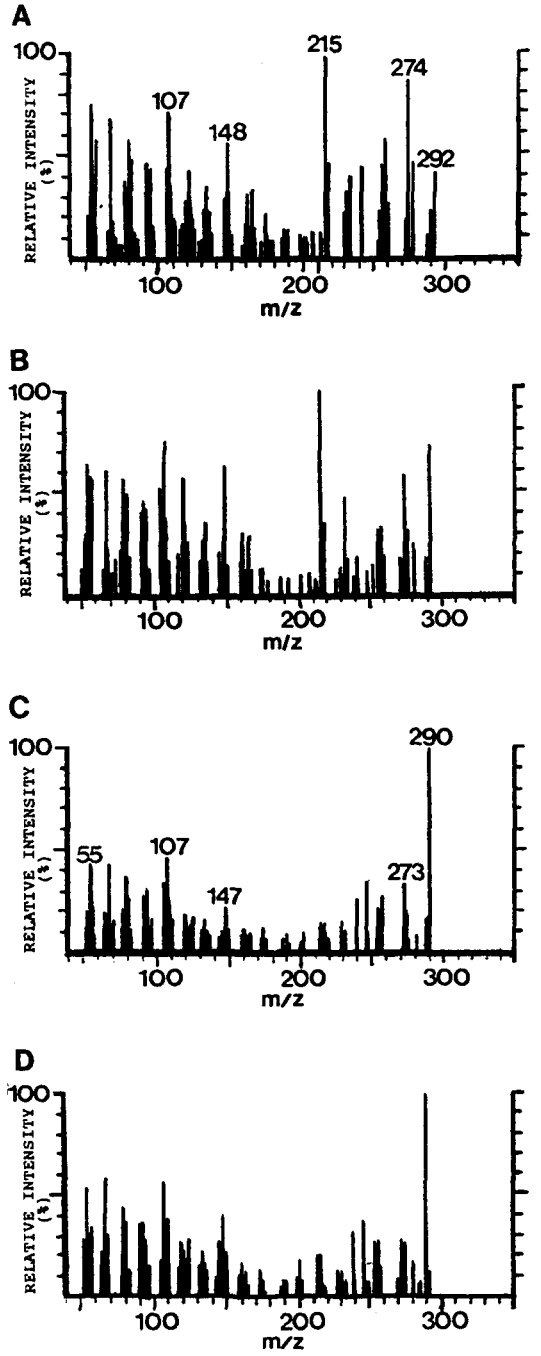

Fig. 6. Mass spectra of material collected at retention times of 25.5 and 40.5 min from HPLC separation of steroid products. (A) Reference standard of 3β-A. (B) Compound extracted from media of porcine Leydig cells incubated with A as substrate (retention time on HPLC = 25.5 min). (C) Reference standard of epiandrosterone. (D) Compound isolated from testicular venous blood of a mature boar (R_t on HPLC = 40.5 min).

and to identify the steroid by GC–MS. No clear evidence for 3β-A was obtained for the corresponding free steroid fractions. These findings are in agreement with data on steroids in boar testicular tissues [13], where the epimer of greatest abundance was 3β-A, occurring in the sulfoconjugated form only. It is assumed that this epimer is the one identified by GC–MS in our study.

While little or no 3β-A left the Leydig cells as free steroid when [^{3}H]A was used alone, the presence of the aromatase inhibitor (4-OH A) led to a significant reduction in the sulfotransferase activity of these cells; greater amounts of 3β-A were seen in the free steroid fraction. In the present context however, attention is drawn to the pronounced peak at 25.5 min, with lesser amounts of 40.5 min, in both the free and sulfoconjugated fractions.

An even more exaggerated profile was obtained when 1,4 T was included in the medium. Competition from the much higher quantity of 1,4 T resulted in most of the radioactivity from [^{3}H]A metabolism appearing in the steroid sulfate fraction, mainly as the two peaks of interest (25.5 and 40.5 min). Partial characterization of the peaks as 5α-reduced products was attempted successfully. Neither the conjugated nor free steroid fractions showed radioactive peaks for 3β-A and epiandrosterone when a 5α-reductase the inhibitor had been added to the medium.

At present the physiological significance of the secretion of large amounts of 3β-A by porcine Leydig cells remains unknown. Unconjugated 3β-A is an important androgen metabolite of the human testicular–epididymal unit [14], where it was suggested that it might play a specific role in regulating spermato- and spermiogenesis, respectively. Also, 3β-A is among the most potent naturally occurring androgens in stimulating rat prostate [15, 16]. The fact that 3β-A could not be converted back to 5α-dihydrotestosterone in rat prostate organ culture was considered as the basis for a regulatory activity of this steroid [17]. Moreover, it has been demonstrated that pharmacological doses of 3β-A are capable of inducing estrogenic responses in the uterus [18]; thus 3β-A could have significance in relation to the known stimulatory effect of estrogens on the accessory sex organs in the boar [7, 19]. Studies on the possible action of 3β-A in the boar, therefore, must include an examination of steroid sulfatase activity which presumably would be a prerequisite in any responsive tissues.

The limited ability of the boar accessory sex organs to hydrolyse C_{19}, 5-en-3β-ol steroid sulfates *in vitro* contrasts with their good ability to convert estrone sulfate to unconjugated estrogens [20]. A sulfatase capable of acting on dehydroepiandrosterone sulfate is present in the prostate in man [21]. It remains to be seen whether a sulfohydrolase action in the accessory sex organs in the boar can be expressed toward neutral steroid sulfates which are saturated, such as 3β-A sulfate and epiandrosterone. In conclusion, the prominence of 3β-A sulfate, and to a lesser extent of epiandrosterone sulfate, in the steroid secretory profile of porcine Leydig cells makes it necessary to include these compounds in future studies on the endocrine role of the testes in this species.

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