J. steroid Biochem. Vol. 22, No. 3, pp. 371-376, 1985
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0022-4731/85 $3.00 + 0.00
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METABOLISM OF TESTOSTERONE BY HUMAN SEMEN

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(*Received* 10 *August* 1984)

Summary—Following the incubation of human sperm and seminal plasma with $^{13}C_2$-labelled testosterone, the main metabolite, identified by gas chromatography-mass spectrometry (GC–MS), was 4-androstene-3,17-dione. In addition, 6α- and 6β-hydroxytestosterone were identified. The more common metabolites of testosterone were not detected, and it is possible that the high substrate-tissue ratio influenced the result. Incubation of individual sperm and seminal plasma specimens with [^{14}C]testosterone resulted in the identification, by specific activity measurements, of 4-androstene-3,17-dione in almost every specimen but with a widely varying conversion rate. Dihydrotestosterone, which on general grounds was considered a likely metabolite, could not be positively confirmed as such, although in some samples its presence was suspected. Gas chromatography-mass spectrometry was also used to identify steroids in sperm and seminal plasma extracts. Some, but not all the steroids identified as present in such extracts by other investigators, were found. During the course of this work C_{18} Sep-Pak cartridges were successfully used to prepare fractions suitable for SP-Sephadex and TEAP-Lipidex chromatography and subsequent analysis by GC–MS. Their use eliminated the need for purification steps otherwise necessary.

INTRODUCTION

Although a great deal has been known for many years about the biochemistry of semen (Mann[1]), only in the last decade or so has it been established that human semen contains androgenic material. Various workers had previously claimed the presence of steroids in semen, but with the possible exception of dehydroepiandrosterone (DHA) no clear identifications were made. That seminal fluid would contain steroids was considered likely when it was shown that the tubular system of the testis was capable of androgen formation *in vitro* from steroid precursors [2, 3], and this has been confirmed using the sensitive assay methods now available.

Adamopolous *et al.*[4] using a combination of Sephadex LH20 chromatography and competitive protein binding determined the concentration of testosterone in semen from subjects with a normal, subnormal or absent sperm count. Unconjugated testosterone was detected in all specimens, with no significant difference between the groups studied (mean concentration 0.73 ng/ml). Purvis *et al.*[5] measured, by radioimmunoassay, unconjugated pregnenolone, DHA, 4-androstene-3,17-dione (androstenedione), testosterone, dihydrotestosterone (DHT), and oestradiol in seminal plasma from a similar groups of subjects. They found the concentration of DHT, pregnenolone and oestradiol to be significantly lower in azoospermic subjects than in normals, whilst DHT was also low in the seminal plasma of oligospermic subjects. The concentration of testosterone found in normal seminal plasma (176 pg/ml) was considerably lower than that reported by Adamopolous *et al.*[4]. More recently, using mass spectrometry, Reiffsteck *et al.*[6, 7] have identified and determined the concentration in seminal plasma of a number of steroids.

The source of these steroids is variable. Purvis *et al.*[5] found that for testosterone the ratio blood plasma:seminal plasma was 37:1 and it is possible that some testosterone in semen could be derived from blood plasma. Some is almost certainly derived from the accessory glands, whilst DHT is probably mainly of testicular or epididymal origin. Other C_{19} steroids might result from the metabolism of testosterone by enzymes present in semen, but although some metabolic experiments have been performed using semen from farm animals [8, 9, 10], investigation of the metabolic activity of human semen has been neglected.

We report here work undertaken to determine how effectively human semen metabolizes testosterone. In preliminary experiments [^{14}C]testosterone was used; subsequently the substrate was testosterone labelled with $^{13}C_2$. Also reported are some results obtained by mass spectroscopic analysis of semen extracts.

EXPERIMENTAL

[3,4-$^{13}C_2$]Testosterone was purchased from P & S Biochemicals Ltd, Liverpool; its purity was checked by mass spectroscopy, and its ^{13}C content found to be 95%.

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[4-^{14}C]Testosterone (sp. act. 58 mCi/mmol), 5α-dihydro[1,2,4,5,6,7-^{3}H]testosterone (sp. act. 147 Ci/mmol) and [1,2,6,7-^{3}H]androst-4-ene-3,17-dione (androstenedione, sp. act. 80–110 Ci/mmol) were obtained from Amersham International, Amersham, England. Each was periodically checked for purity by thin layer chromatography on polysilicic acid gel impregnated glass fibre sheets (ITLC, Gelman Hawksley, Northampton, England), followed by scanning in a radiochromatogram scanner (Packard, Model 7201).

Radioactivity was counted in a liquid scintillation counter (Packard, Tri-Carb). Counting efficiency was 90% for ^{14}C and 50% for ^{3}H.

Non-radioactive steroids were obtained from Professor D. N. Kirk (MRC Steroid Reference Collection) or from Sigma Chemical Co. Ltd, Poole, Dorset, England.

SP-Sephadex LH20 (sulphohydroxypropyl-Sephadex LH20) was prepared from Sephadex LH20 by the method of Axelson and Sjövall[12], and TEAP-Lipidex 5000 (triethylaminohydroxypropyl-Lipidex 5000) was prepared from Lipidex 5000 (Packard Instrument Co.) as described by Ellingboe, Almé and Sjövall[13], Axelson and Sjövall[14, 15] and Tetsuo *et al.*[16].

Florisil (B.D.H., Poole, England) was purified, and activated by heating for 4 h at 600°C [17].

Sep-Pak C_{18} cartridges were obtained from Waters Associates Ltd, Hartford, Cheshire, England.

Solvents (B.D.H., Poole, England) were analytical grade and were distilled before use.

Semen samples were obtained from men undergoing, with their wives, tests for infertility. None of the samples used had a sperm count of less than 20×10^{-6}/ml. The samples were stored at 4°C for up to 4 weeks. Sperm and seminal plasma fractions were obtained by centrifugation. The incubation buffer was a 0.05 M phosphate-saline buffer, pH 7.2, containing 0.1% w/v penicillin.

Incubation conditions

The sperm fraction from pooled semen was suspended in a volume of buffer equal to the volume of seminal plasma. Both fractions were incubated in flasks containing [$^{13}C_2$] testosterone (2 mg) and [1,2,6,7-^{3}H]testosterone (3.34 μCi, 9 ng) dissolved in ethanol (0.5 ml). A control flask contained buffer and substrate only. The incubation period was 4 h, in air and at 37°C. Co-factors were not added. At the end of this time acetone (8 vol) was added to each flask, which were then refrigerated, to hasten complete protein precipitation.

Extraction of steroids from sperm and seminal plasma

(a) Seminal plasma (50 ml) was added to acetone (8 vol), and the resulting precipitate removed by filtration. The filtrate was evaporated to dryness. The residue was partitioned between 70% methanol and hexane, and after removal of the methanol the aqueous solution was adjusted to pH 3 and extracted with ether–ethyl acetate (1:1 0.5 vol × 3) and ethyl acetate (0.5 vol × 1). The combined extracts were washed with concentrated carbonate solution (8% $NaHCO_3$, pH 10.5) and water.

The corresponding sperm fraction was stirred in acetone (50 ml) and filtered. The filtrate was treated as for seminal plasma.

Both extracts were dissolved in benzene and chromatographed on a column of florisil (10 × 2 cm). Free steroids were eluted by benzene (160 ml), benzene–ethyl acetate, 1:1 (80 ml) and ethyl acetate (80 ml) [18]. The combined eluates were further purified for GC–MS by chromatography on SP-Sephadex and TEAP-Lipidex.

SP-Sephadex chromatography

SP-Sephadex, stored in the Na^+-form was converted to the H^+-form by washing with 0.2 M HCl (100 ml), and a column prepared in 72% aqueous methanol (5.0 × 150 mm). The material to be chromatographed was put on the column in aqueous methanol (0.5 ml methanol and 2.5 ml 75% aqueous methanol). The column was washed with 72% aqueous methanol (13 ml), and the combined effluents applied to a TEAP-Lipidex column [16].

TEAP-Lipidex chromatography

TEAP-Lipidex was converted before use from the acetate form (in which it was stored) to the OH^--form by washing with the following solvents (100 ml each): 0.2 M NaOH in 72% ethanol, 20% ethanol and ethanol. The washed gel was allowed to swell for 1 h in 72% methanol (10 ml) and then used to prepare a column (5.0 × 150 mm) through which the SP-Sephadex eluate was percolated. The effluent was added to the first of three fractions collected as follows—(F1) neutral steroids eluted with 72% methanol (8 ml), (F2) non-polar lipids eluted with methanol–chloroform, 80:15,v/v (10 ml), (F3) phenolic steroids eluted with methanol–chloroform, 80:15, v/v (8 ml) saturated with CO_2. A pressure of CO_2 was applied to the column to give a flow rate of about 20 ml h^{-1} [16]. F1 fractions were further analysed by gas chromatography–mass spectrometry (GC–MS) [see below].

(b) Later semen samples were separated into plasma and sperm, and after precipitation, and removal of acetone, the aqueous solution was chromatographed on a primed C_{18} Sep-Pak cartridge, and steroids eluted with methanol (2 ml) [19]. The 70% methanol–hexane partition and florisil chromatography were omitted, the eluate from the Sep-Pak cartridge being chromatographed directly on SP-Sephadex and then TEAP-Lipidex.

Extraction after incubation

This was performed as for the extraction of steroids from sperm and seminal plasma, but after florisil chromatography, the free fraction from each incubation was chromatographed as an 18 cm strip

on polysilicic acid gel glass fibre sheets, twice in the system methylene chloride-dioxan 97:3. Unchanged testosterone was located under u.v. light. Three areas were cut from each chromatogram: (a) from the origin to the testosterone area (Pre-T), (b) unchanged testosterone (T), (c) testosterone to the solvent front (Post-T). Each was eluted with methanol. After evaporation to dryness the residue was partitioned between water and ethyl acetate to remove non-steroidal material extracted from the glass fibre sheets. The steroids were then chromatographed on SP Sephadex and TEAP-Lipidex.

Gas chromatography–mass spectrometry

The mass spectrometer was a VG Micromass 7070F coupled to a Pye Unicam 204 gas chromatograph and a Finnigan Incos data system. A fused silica capillary column (25 m × 0.2 mm i.d. OV-1) with a column temperature of 24°C, and a carrier gas pressure (helium) of 10 p.s.i. was used. Injection was by means of an on-column injector (OCI-3, from SGE (U.K.) Ltd). The column was interfaced to the mass spectrometer by inserting its end directly into the ion source. Mass spectra were recorded every 2.25 s, under the following conditions: accelerating voltage 4 kv, emission current 200 μA, electron beam energy 70 eV, ion source temperature *ca* 220°C. The GC inlet line was maintained at 250°C.

Before GC–MS analysis, each fraction was acetylated by dissolving in pyridine–acetic anhydride (1:1 200 μl) and heating at 55°C for 1 h. The solvents were removed under nitrogen and the acetylated material dissolved in ethyl acetate, usually 50 μl. Volumes of 1–4 μl were used for injection. Standards (e.g. testosterone, hydroxytestosterone) were treated in the same way.

RESULTS

No incubation

Androstenedione, DHA and pregnenolone were identified by mass spectroscopy in extracts of both sperm snd seminal plasma. Additionally, DHT was detected in the sperm extract. These steroids were present in the TEAP-Lipidex F1 fraction. Also in this fraction prostaglandins were invariably found. In the non-polar lipid fraction (F2) of both sperm and seminal plasma squalene, cholesterol and 7-dehydrocholesterol were characterized. These compounds were also present in F3 (Phenolic fraction) and must represent a spill-over from F2.

A 15 ml pool of semen was not centrifuged but precipitated directly by the addition of acetone. After removal of solvent and the addition of water (10 ml) the aqueous solution was passed through a Sep Pak C_{18} cartridge.

After washing with water, steroids were eluted with methanol (2 ml), the volume made up to 7.5 and 1.0 ml (= 2 ml semen) chromatographed on SP-Sephadex and TEAP-Lipidex. Fraction F1 was then chromatographed on Sephadex LH20 (2 g in col. 10 mm i.d.) using the system iso octane–chloroform–methanol, 85:5:5. Fractions (1 ml) were collected and pooled as follows, 4–10 (A), 11–16 (B), 17–23 (C), 24–28 (D) and 29–36 (E); these divisions were based on the results of chromatographing standard steroids. The fractions were then analysed by GC–MS using the selective ion monitoring technique. Identifications were made as follows. Fraction A showed a peak with the correct retention time for progesterone. A number of other larger peaks were present, but not identified; fraction B, androstenedione and pregnenolone were identified; fraction C, a large peak at the correct retention time for DHA and a small peak at the correct retention time for DHT were present: fraction D, a small peak at the correct retention time for testosterone appeared as a shoulder on a larger peak, and positive identification was not certain; fraction E, no peak apparent (17-hydroxyprogesterone [7] would appear in this fraction if present).

After incubation

The labelled steroids identified by mass spectroscopy following incubation of sperm and seminal plasma with [$^{13}C_2$]testosterone were androstenedione, 6α-hydroxytestosterone and 6β-hydroxytestosterone. Other normal metabolites of testosterone were either not found, or in amounts no greater than found in the control incubation.

DISCUSSION

A qualitative study by capillary gas chromatography–mass spectrometry of fractions prepared from sperm and seminal plasma showed the presence of androstenedione, DHA and pregnenolone in both, and a small amount of DHT in the sperm extract. Each steroid was characterized, as its acetate, by identity of retention time and mass spectrum with an authentic standard. Identity of mass spectra was established by library searching [20] using a sublibrary of reference mass spectra of steroids and their derivatives. In addition to these compounds, progesterone was identified by the selective ion monitoring technique in an extract of whole semen. Other compounds stated to be present in seminal plasma (testosterone, cortisol, oestradiol, 17-hydroxyprogesterone) were not identified under the conditions used.

In order to be certain that compounds isolated after incubation experiments were metabolites of the substrate, testosterone containing the stable isotope carbon-13 ([3,4-$^{13}C_2$]testosterone) was used. As long as the stable isotope is at a site that does not affect, and is not affected by, the metabolic process, then compounds containing the isotope may be considered to be derived from the substrate. Compounds labelled with $^{13}C_2$ can readily be distinguished from unlabelled compounds by mass spectrometry. A recent report

has stated that [^{13}C]testosterone is metabolically equal to [^{12}C]testosterone [21].

Following incubation of sperm and seminal plasma with $^{13}C_2$-labelled testosterone, labelled androstenedione, 6α-hydroxytestosterone and 6β-hydroxytestosterone were identified as metabolites by capillary GC–MS. Androstenedione was the major metabolite. Some [$^{13}C_2$] DHT was formed, but the amount was no greater than that found in the control incubation. The labelled 6-hydroxytestosterone derivatives had the same retention times as reference samples of acetylated 6α- and 6β-hydroxytestosterone, and the mass spectra matched well, having regard to the 2 mass unit difference resulting from the incorporation of $^{13}C_2$. Figure 1 shows the mass spectra of labelled 6α-hydroxytestosterone (a) and the authentic unlabelled compound (b), both as acetates.

Testosterone is extensively metabolised in many tissues, but not usually to 6-hydroxylated derivatives. The usual reactions include oxidation, followed by reduction, with the subsequent formation of androsterone and aetiocholanolone, both common urinary metabolites of testosterone. Alternatively, or in addition, reduction may give rise to isomeric androstanediols, principally 5α- and 5β-androstane-3α,17βdiols. These reactions result in compounds biologically less active than testosterone; the more active 5α-dihydrotestosterone is formed by target tissues, such as ventral prostate [22].

However, the introduction of a 6-hydroxyl group into the testosterone molecule is not unknown, and

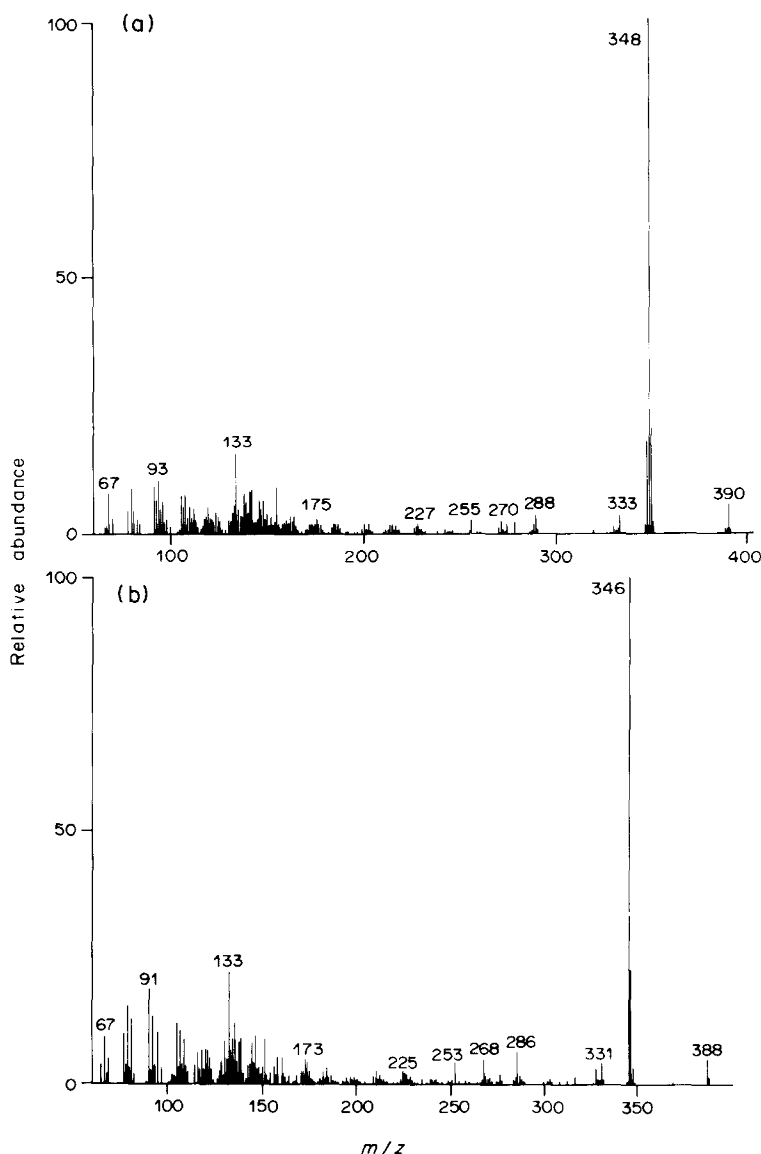

Fig. 1. Mass spectra of (a) [3,4-$^{13}C_2$]6α-hydroxytestosterone acetate ($M^{+\cdot}$, *m/z* 390; $(M\text{-}CH_2CO)^{+\cdot}$, *m/z* 348) observed in extract following incubation of sperm with [3,4-$^{13}C_2$]testosterone, and (b) authentic 6α-hydroxytestosterone acetate showing 2 mass unit difference ($M^{+\cdot}$, *m/z* 388; $(M\text{-}CH_2CO)^{+\cdot}$, *m/z* 346).

Table 1. Androstenedione formation after incubation of sperm and seminal plasma with [4-^{14}C]testosterone

	Sperm				Seminal plasma			
Spec. no.	Total sperm count $\times 10^6$	Sperm incubated $\times 10^6$	% Conversion	nmol androstenedione per specimen	Total volume (ml)	Volume incubated (ml)	% Conversion	nmol androstenedione per specimen
1	44	14	26.2	31.0	7.0	2.3	3.2	4.53
2	52	40	0.94	0.48	2.6	2.0	0.20	0.10
3	72	48	40.0	24.0	3.0	2.0	0.00	0.00
4	92	81	0.01	0.01	4.0	3.5	0.15	0.07
5	100	80	0.11	0.02	2.5	2.0	0.17	0.08
6	112	106	83.3	34.7	3.5	3.3	3.2	1.33
7	140	100	2.6	1.43	3.5	2.5	0.69	0.39
8	187	145	4.8	2.46	5.5	4.3	0.11	0.06
9	203	171	1.6	0.75	4.5	3.8	0.27	0.13
10	230	137	0.08	0.05	3.2	1.9	0.03	0.02
11	250	182	0.02	0.01	5.5	4.0	0.03	0.02
12	322	279	0.17	0.08	5.2	4.5	0.15	0.07
13	328	271	0.08	0.04	4.0	3.3	0.04	0.02
14	342	257	0.04	0.02	6.0	4.5	0.04	0.02
15	388	217	0.67	0.47	2.5	1.4	0.03	0.02
16	390	208	0.13	0.10	3.0	1.6	0.12	0.09
17	416	327	0.03	0.01	4.2	3.3	0.15	0.07
18	426	142	0.42	0.50	3.0	1.0	0.00	0.00
19	455	232	1.0	0.79	4.5	2.3	0.00	0.00
20	488	365	0.06	0.03	4.0	3.0	0.04	0.02
21	494	416	0.00	0.00	9.5	8.0	0.10	0.05
22	522	522	0.30	0.12	4.5	4.5	0.42	0.17
23	584	320	0.12	0.09	4.0	2.2	0.02	0.01
24	616	462	18.7	0.98	4.0	3.0	0.90	0.47
25	662	440	2.4	1.44	4.5	3.0	0.36	0.21
26	698	504	0.08	0.05	4.5	3.3	0.09	0.05

several such compounds have been isolated from bull testes [23]. Both 6α- and 6β-hydroxytestosterone have been formed after incubating testosterone with preparations from adult rat testes [24] and 6β-hydroxytestosterone after incubation with human fetal liver microsomes [25]. Following the infusion of testosterone and testosterone phosphate to a woman, 6β-hydroxytestosterone was isolated from the urine [26]. In most incubations of tissue with steroids, the nature of the final metabolites can be influenced by a number of factors in addition to the enzymes present e.g. the addition or otherwise of co-factors, the length of time of incubation, temperature, pH, and the ratio of substrate to tissue. Metabolic products also accumulate, and tend to inhibit their own further production. The 6β-hydroxylation pathway is stated to assume great significance *in vivo* after the administration of high doses of steroids [25], and possibly the high substrate-tissue ratio in this incubation influenced the metabolic pathway, resulting in 6-hydroxylation. It may be assumed that sperm and seminal plasma contain a 6-hydroxylase, but the significance of 6-hydroxylation in this experiment is difficult to determine; it may be a step in androgen inactivation [27].

The formation of androstenedione as the major metabolite when human sperm or seminal plasma is incubated with testosterone, taken with results from animal experiments, in which androstenedione was also the principal metabolite [8, 9, 10, 11] suggests that this reaction may be universal among mammals, although androstenedione is not necessarily the major metabolite when other tissues are incubated with testosterone [28].

Before [$^{13}C_2$]testosterone was available, individual sperm and seminal plasma samples were incubated with [4-^{14}C]testosterone (2μCi, sp. act. 58 mCi/mmol) and 5α-dihydro[1,2,4,5,6,7-^{3}H]testosterone (16,000 dpm, sp. act. 147 Ci/mmol) principally to see if DHT was a metabolite. There was some evidence of DHT formation when testosterone was incubated with sperm, but generally the amounts were too small to state with confidence that it was produced. Androstenedione however, identified by crystallization to constant $^3H/^{14}C$ ratio (after adding [1,2,6,7-^{3}H]androstenedione) was formed by almost every sperm sample, (Table 1). The conversion rate varied widely between samples and was generally less than 1%. The seminal plasma conversion rate was even smaller.

Thus, although DHT is a major metabolite when testosterone is incubated with some tissues it is not so when testosterone is incubated with sperm, suggesting that its formation by this means has no great physiological importance.

Acknowledgements—We thank the Mersey Region Health Authority Research Committee for providing financial support for this study, and Miss M. E. Holliday for skilled technical assistance.

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