IDENTIFICATION OF A UNIQUE SERTOLI CELL STEROID AS 3α-HYDROXY-4-PREGNEN-20-ONE (3α-DIHYDROPROGESTERONE: 3α-DHP)

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ABSTRACT

An allylic steroid produced from progesterone by rat Sertoli cells and which does not appear to have been described previously as a product of gonadal or adrenal tissues has been isolated and identified as 3α-hydroxy-4-pregnen-20-one (3α-dihydroprogesterone; 3α-DHP). 3α-DHP appears to be a reactive molecule which is easily oxidized or dehydrated and its identification was possible by a combination of microchemical procedures, derivative formation, specific enzyme reaction, TLC, GC, HPLC, spectrophotometry and mass spectrometry. The biological functions of this Sertoli cell steroid are not known, but it is suggested that 3α-DHP is more than a metabolic waste product because (a) its production rate varies with age and is highest at the onset of meiosis, and (b) there appear to be specific receptors for it in the testis.

INTRODUCTION

The vertebrate male gonad consists essentially of two compartments: the seminiferous tubules (or lobules) which contain the germ cells, and the interstitial tissue which occurs between the tubules. Large, polymorphic cells, called Sertoli cells, line the inside of the tubules and the germ cells are surrounded or "embraced" by the Sertoli cells during the process of spermatogenesis. No blood enters the tubule compartment and the Sertoli cells are believed to provide all the necessary nutrients and milieu required for spermatogenesis. Although the interstitial region has long been known to be the major source of androgenic steroids in the mature male, Sertoli cells have also been shown, in recent years, to be capable of steroid synthesis (1-3). We have shown that the steroidogenic capacity of Sertoli cells changes with age, with a marked peak in conversion of testosterone (17β-hydroxy-4-androsten-3-one) and progest-

erone to a number of C_{21} and C_{19} steroids (4-6). Nine of the metabolites of progesterone (constituting about 88% of the mass of the total metabolites) were recently identified (7). However, one major metabolite which constituted over 7% of the amount of all the progesterone metabolites, remained unidentified. The present report describes the evidence for the identity of this metabolite as 3α-hydroxy-4-pregnen-20-one (3α-DHP), a steroid which does not appear to have been described previously as a product of gonadal or adrenal tissues. Evidence is also presented which indicates that 3α-DHP is produced by Sertoli cells but not by Leydig cells and that the synthesis changes with age.

MATERIALS AND METHODS

Animals. Sprague-Dawley strain rats from our own colony (original breeders purchased from Bio Breeding Laboratories of Canada, Ltd., Ottawa) were maintained on a 14-h light, 10-h dark photoperiod, at 24°C and fed Purina Rat Chow and water ad libitum. Males at various ages were employed. In some instances, males had been irradiated in utero (7) in order to increase the yield of Sertoli cells.

Chemicals. Inorganic salts and organic solvents were of analytical or spectral grade. Solvents used for extraction were glass distilled prior to use. The [4-^{14}C]-progesterone (56.95 μCi/μmole) and [1,2,6,7-^{3}H]-progesterone (105 μCi/mole) were purchased from New England Nuclear and were purified by thin layer chromatography (TLC; chloroform/ether, 10:3) prior to use. Over 100 steroid standards were available for this study and these had been obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.), Steraloids Inc., (Wilton, N.H., U.S.A.), Makor Chemical Ltd., (Jerusalem), or the Steroid Reference Collection (M.R.C. London, U.K.).

Sertoli Cell Preparation and Incubation. Rats at a particular age were weighed and then sacrificed by cervical dislocation. Testes were removed aseptically, washed, and Sertoli cells were isolated by sequential enzymatic disaggregation (8) and seeded in 35 x 10 mm or 60 x 15 mm Corning plastic petri dishes at a concentration of approximately 1.5 x 10^6 and 4.5 x 10^6 cells respectively per small and large dish. In order to maximize production of metabolites, the culture medium previously described (8), was supplemented with FSH (Sigma No. F-8001, porcine) at 5 μg/ml and [^{14}C]-progesterone (2.681 μCi/μmole) at 0.015 μCi/ml. The cells were incubated at 32°C in 3 ml (small dishes) or 5 ml (large dishes) of medium and the medium was changed every 20-48 hours and extracted for steroids. For the age-related studies, Sertoli cells were pre-

cultured for 72 h as previously described (6,7,8); after 72 h the medium was replaced with fresh medium containing [4-^{14}C]-progesterone (0.65 μCi/11.56 nmoles/dish) and cells were incubated at 32°C for an additional 1,3,6,20 or 48 hours (7). The reactions were terminated by mixing cells and/or medium in an extraction tube containing 2x the volume of ether/chloroform (4:1).

Leydig Cell Preparation and Incubation. Testes were detunicated, sliced at 2-3 mm, placed in a 50 ml Erlenmeyer flask and incubated at 37°C in Earle's salt solution (about 3 g testis weight/25 ml) containing 0.25 mg/ml collagenase (Sigma Type I, No. C-0130; 165 units/mg). After 20 min. of incubation (with periodic agitation) the flasks were placed on ice and the salt solution was decanted through a stainless steel sieve (0.5 mm). The settled tubule portion in the incubation flask was washed several times with 10-20 ml Earle's salt solution, the washes, containing the interstitial cells, were decanted through the sieve and combined. Cells which had passed through the sieve were collected by centrifugation at 80 x g (5 min) and resuspended in a mixture consisting of 15 ml of Percoll (Pharmacia, Lot No. 4634) and 15 ml of Earle's salt solution (containing 1.8% NaCl). The mixture was then centrifuged at 20,000 x g (Sorvall SS 34 fixed angle rotor) for 35 min. The cells formed several bands. On the basis of a positive histochemical reaction for 5α/β-3β-hydroxysteroid oxidoreductase activity (9), the band just above the red blood cells was found to be highly enriched in Leydig cells. This band of cells was collected with a 16 gauge flat point needle and syringe; it was then diluted 10 fold with Earle's salt solution, the Leydig cells were collected at 80 x g (5 min) and washed several times with Earle's salt solution.

Leydig cells were incubated in either the Sertoli Cell Medium (as above) or Medium 199; the medium was supplemented with NADPH (Sigma; 1.6 mg/ml) and NAD (Sigma; 10 mg/ml) and contained [4-^{14}C]-progesterone (0.65 μCi/11.56 nmoles/culture tube). Incubations occurred for 15, 30, 60 or 120 min at 34°C with gentle shaking in a water bath. Leydig cells were also seeded in petri dishes and cultured for 20 h at 32°C in the same manner as Sertoli cells. Incubations were terminated by thorough mixing with 6-10 ml ether/chloroform (4:1).

Histochemistry and Cytology. The purity of Sertoli and Leydig cells and the number of cells in each preparation was determined by cytological and histochemical criteria (8,9). On the basis of these criteria, the Sertoli cell preparations were about 98% pure and the Leydig cell preparations consisted of 80-94% Leydig cells.

Steroid Extraction and Identification. The samples were treated as previously described (7). Briefly, each sample was extracted three times with 2-4 volumes of ether/chloroform (4:1), concentrated under nitrogen and spotted on the lower right hand corner of a 20 x 20 cm silica gel GF thin layer plate (Fisher Redi/Plate, 0.25 mm). Each plate was run 2 times in System I (chloroform/ether, 10:3), then turned 90°and run 2 times in System II (hexane/ethyl acetate, 5:2). The plates were then

apposed to Kodak Medical X-Ray film (X OMAT R Film) for a 7-10 day exposure, or the radioactive metabolites were localized on the TLC plate via a β-spark chamber (Berthold Beta Camera LB 291). The radioactive spots were numbered and the metabolite of concern in this communication was designated No. 4 in a previous publication (7). Figure 1a shows the position of No. 4 relative to several other, identified, metabolites run in the above TLC systems. The Rf value of the No. 4 spot was compared to those of 109 authentic steroids chromatographed in the same systems. The No. 4 spot was scraped from the TLC plates, eluted with methanol or ether/chloroform (4:1), concentrated under N_2 and stored in benzene or methanol.

Chemical Alterations of Metabolite No. 4

Aliquots (about 25,000 dpm) of metabolite No. 4 were treated in the following ways. Reductions were performed with $NaBH_4$ in 0.1-0.2 ml of either methanol or t-butanol (at 4°C for 1 hr or room temperature for 2 hr, respectively), or with 0.1 ml tetrahydrofuran containing 0.5 M 9-borabicyclo[3.3.1]nonane (Aldrich Chem. Co.; overnight at room temperature). Oxidations were performed either in 0.2 ml of a 1:1 mixture of acetic acid and aqueous CrO_3 (2% w/v) or in 0.1 ml dioxane containing 0.1 mg of 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ; Eastman), for 2 hr at room temperature or overnight at 4°C. Following the reduction or oxidation reactions, water was added and the steroids were extracted with ether or ether/chloroform (4:1). Acetylations occurred overnight at room temperature in 0.2 ml pyridine/acetic anhydride (1:1) mixture. Silylation was performed in 0.1 ml of either of the following mixtures: (a) bis(trimethylsilyl) acetamide/pyridine (1:10), (b) bis(trimethylsilyl) acetamide/trimethylchlorosilane/pyridine (5:1:50), or (c) n-methyl-n-trimethylsilyl-trifluoroacetamide (MSTFA; Pierce Chem. Co.); reactions in mixture (a) or (b) were allowed to proceed overnight at room temperature and in mixture (c), for 2 hr. The samples were then dried down under N_2 at 40°C and redissolved in methanol. Dinitrophenylhydrazones (DNPH) were formed by dissolving steroid in 0.1 ml methanol (containing 10 µl HCl), adding 0.1 ml methanol containing 0.3 mg of 2,4-dinitrophenylhydrazine and heating the mixture for 15 min at 50°C; the sample was then dried down under N_2 at 40°C and dissolved in methanol.

Preparation of Enzymes

3β-hydroxysteroid oxidoreductase (3β-HSO). The 3β-HSO was prepared, from the adrenals of 10 female Sprague-Dawley rats aged 60-90 days, by the method previously described (10) using NaCl (1.0M) to solubilize the enzyme from the microsomes (10) and the method of (11) to desalt the enzyme solution with Sephadex G-25-300.

3α-hydroxysteroid oxidoreductase (3α-HSO). The 3α-HSO was prepared from about 40 g of liver tissue from female rats aged 60-90 days. The tissue was minced with scissors and rinsed several times with Solution B (Na_2HPO_4, 0.8 mM; NaH_2PO_4, 0.2 mM; sucrose, 250 mM; EDTA, 1.0 mM; pH 7.3). The tissue was homogenized in Solution B (20% w/v) using a Poly-

tron with PT-10 generator for 30 sec at 23,000 rpm. The homogenate was centrifuged at 12,000 x g for 5 min; the supernatant was centrifuged at 25,000 x g for 10 min and this supernatant was then centrifuged at 200,000 x g for 2 hr. The 200,000 x g supernatant was used for the 3α-HSO reactions.

20α-hydroxysteroid oxidoreductase (20α-HSO). The 20α-HSO was obtained from ovaries of rats 41-90 days old by a modified procedure of (12). Minced ovaries were homogenized with a Polytron (23,000 rpm for 30 sec) in Solution C (Tris-HCl buffer, 0.1 M, containing 0.9 mM EDTA, 1.0 mM cysteine, 10 mM nicotinamide, and 0.042 mM progesterone, pH 8.0 at 4°C). The homogenate was centrifuged at 25,000 x g for 30 min and the supernatant was treated with an equal volume of 4 M ammonium sulfate (in 0.1 M Tris buffer, pH 8.0). The ammonium sulfate was added over a period of 30-60 min and the solution was allowed to stand for 2 hr at 4°C. The precipitated protein was removed by centrifugation (10 min at 12,000 x g) and the supernatant was again treated slowly with an equal volume of 4 M ammonium sulfate and allowed to stand overnight at 4°C. The precipitate was collected by centrifugation (10 min at 12,000 x g), dissolved in Tris buffer (0.1 M, pH 8.0), desalted in Sephadex G-25 (as for 3β-HSO above) and stored at -70°C.

Enzymatic Reactions. Enzymatic conversions of No. 4 were monitored either by spectrophotometric methods or by employing TLC, HPLC, autoradiography and scintillation spectrometry.

(a) Spectrophotometric measurements. About 100 ng of No. 4 (in 100 µl methanol) was placed in a one ml semi-micro cuvette (10 mm light path) to which 0.85 ml of Na/K phosphate buffer (0.05M, pH 8.3) containing 50 µg thionicotinamide adenine dinucleotide phosphate (thioNADP) was added. After 5 min at 30°C, the reaction was started with 50 µl of the 3α-HSO, 3β-HSO or the 20α-HSO enzyme preparation. The change in absorption was followed at 395 nm in a temperature-controlled (30°C) Unicam SP 1800 spectrophotometer with AR 25 linear recorder. After 2 min the reaction mixture was extracted with 6 ml ether/chloroform (4:1). The ether/chloroform was evaporated under nitrogen and the extract dissolved in 100 µl HPLC grade methanol. Twenty µl of extract was injected on an Altex/Beckman HPLC system with C-18 column. Elution was with a water/methanol mixture (28:72) at 1.5 cc/min. One min fractions were collected in scintillation vials and following addition of 10 ml dioxane-based scintillation solution (dioxane containing 5g of 2,5-diphenyloxazole and 100g naphthalene per liter of solution) the radioactivity was measured in a Beckman LS 100 or LS 255 liquid scintillation spectrometer.

(b) Chromatographic and radioactive methods. In separate incubation tubes, approximately 100 ng of labelled No. 4 (12,000 dpm ^{14}C and 30,000 dpm ^{3}H) was reacted with 25 µl of either 3α-HSO (2.24 enzyme units), 3β-HSO (4.54 enzyme units) or 20α-HSO (0.51 enzyme units) in a medium consisting of 25 µl methanol, 0.3 ml phosphate buffer (0.05 M, pH 8.3) and 100 µg NADP or NAD. The reaction was initiated by addition of

enzyme and was carried out for 2 min at 25°C. Control tubes lacked enzyme. The reaction was terminated by shaking with 3 ml ether/chloroform (4:1). The aqueous phase was extracted 2 more times with 3 ml ether/chloroform (4:1), the extracts were dried down under N_2, spotted in separate lanes, together with 10 µg progesterone and 20α-hydroxy-4-pregnen-3-one (20α-DHP), on a 20 x 20 cm silica gel GF thin layer plate (Fisher Redi/Plate, 0.25 mm) and the plates were run 2x in System I. The progesterone and 20α-DHP were located by UV fluorescence and the radioactivity was localized by Berthold β-Spark Chamber and by autoradiography (7). The radioactive spots were scraped from the plates, the gels extracted with ether/chloroform (4:1) and methanol, and aliquots were then run on HPLC and/or crystallized with authentic progesterone.

Gas Chromatography. A Hewlett-Packard 5830A gas chromatograph equipped with two 150 cm (4 mm or 2 mm) columns packed with either 3% OV-210 on Gas Chrom Q, 80/100 mesh, or with 3% SE-30 ultraphase on Chromosorb G-HP, 100/120 mesh was used with flame ionization detectors and a glass stream splitting device, as previously described (7).

A Hewlett-Packard 5840A gas chromatograph with a 150 cm (2 mm) column packed with 3% OV-17 Chromosorb G-HP, 80/100 mesh and equipped with electron capture detector was employed for the detection of heptafluorobutyrates, chloroacetates and 2,4-dinitrophenylhydrazones of No. 4 and of standard steroids. The column temperature was 210°C and argon containing 5% methane was the gas phase.

High Pressure Liquid Chromatography (HPLC). A Beckman Model 332 gradient liquid chromatograph with Altex Model 420 microprocessor and Model 155 variable wavelength detector was interfaced with a Hewlett-Packard 5840A GC terminal and was used with an Altex Ultrasphere ODS (C_{18}) column and a Whatman ODS guard column. Commonly, a liquid phase consisting of 72% methanol and 28% water was employed, but other proportions and acetonitrile/water combinations were also used. A wavelength of 240 nm was employed for the detection of 4-ene-3-keto steroids and 206 nm for steroids with 5α-reduced or 3-hydroxy configurations.

Mass Spectrometry. Samples were run at different temperatures on direct probe at 70eV on a Varian MAT 311A GC/Mass Spectrometer (3% SE 30 at 230°C).

RESULTS AND DISCUSSION

The metabolites of [^{14}C]-progesterone produced by isolated Sertoli and Leydig cells and separated by 2-dimensional TLC are shown respectively in Figs. 1a and 1b. The autoradiograph of the Sertoli cell meta-

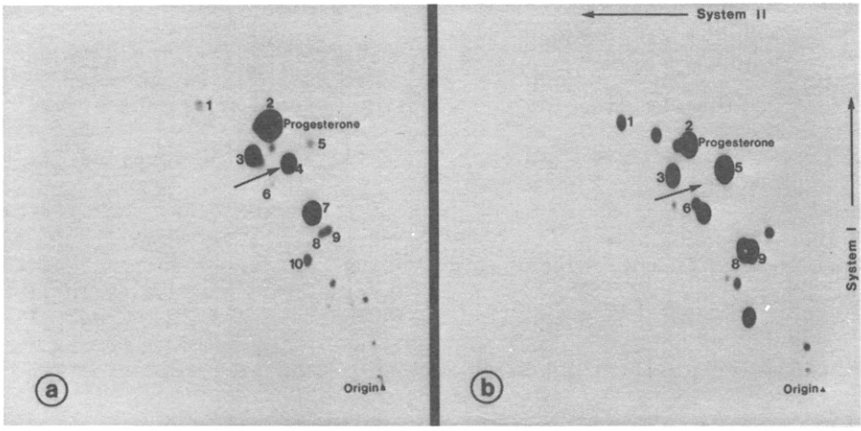

FIGURE 1. Autoradiographs of TLC plates showing the results of incubating [^{14}C]-progesterone, for 20 h, with either (a) Sertoli cells from 17 day old rats, or (b) Leydig cells. The autoradiographs are representative of several hundred obtained between 1978 & 1981. Each culture contained about 1.5 x 10^6 cells. The extracts were spotted on the lower right hand corner and the plates were run 2x in System I and 2x in System II in the direction indicated. The metabolites in (a) are numbered as in a previous publication (7) where No. 4 remained unidentified. Note the absence of a metabolite comparable to No. 4 in the Leydig cell extract (arrow).

bolites (Fig. 1a) indicates the metabolite of interest and referred to as No. 4. It has an Rfp (Rfp = $\frac{\text{Rf of substance}}{\text{Rf of progesterone}}$) of 0.77 when run 2x in System I and 0.82 when run 2x in System II. It can be seen from Fig. 1b that isolated Leydig cells incubated under identical conditions with [^{14}C]-progesterone, do not produce a compound which behaves the same on TLC as the No. 4 from Sertoli cells.

Metabolite No. 4 exhibited a mobility in the TLC systems which was similar but not identical to a number of known steroids in our library of reference standards (Table 1). When [^{14}C]-labelled No. 4 was subjected to gas chromatography on a OV-210 column (230°C, 35 cc N_2/min),

radioactivity collected on the splitting device emerged relatively early (Rtp = $\frac{\text{Retention time of substance}}{\text{Retention time of progesterone}}$ = 0.2 to 0.39). The time of emergence of radioactivity coincided with the retention time of several known steroid standards (Table 1). However, when ^{14}C-labelled No. 4 was crystallized with about 20 mg of each of these steroids, radioactivity did not remain in the crystals, indicating that No. 4 was none of these.

An aliquot of [^{14}C] labelled No. 4 was oxidized with CrO_3. The migration of the oxidized [^{14}C]-No. 4 on TLC and the retention time (Rt) during GC on OV-210 and SE-30 columns now coincided with authentic pro-

Table 1. Relative mobilities and retention times of steroid standards which behave approximately like metabolite No. 4 in TLC Systems I and II[a]

Steroid	Rfp[b] System I	Rfp[b] System II	Rtp[c] (OV 210)
No. 4	0.78	0.82	0.2 - 0.39[d]
3β-hydroxy-5-pregnen-20-one	0.8	0.9	0.36
3β-hydroxy-5α-pregnan-20-one	0.76	0.85	0.4
20α-hydroxy-5β-pregnan-3-one	0.75	0.89	0.49
17-hydroxy-5α-pregnane-3,20-dione	0.82	0.93	1.02
3β-hydroxy-5,16-pregnadien-20-one	0.74	0.8	0.32
3β-hydroxy-5-androsten-17-one	0.74	0.74	0.27
4-androstene-3,17-dione	0.89	0.74	0.79
17β-hydroxy-5α-androstan-3-one	0.75	0.87	0.35
3α-hydroxy-5α-androstan-17-one	0.68	0.8	0.28
3β-hydroxy-5α-androstan-17-one	0.69	0.73	0.31
estradiol-17β	0.70	0.83	0.22

[a] Among our collection of over 100 steroid standards, only the steroids listed in the table behaved approximately like No. 4 in the TLC systems employed.

[b] Relative mobility in relation to the mobility of progesterone when run 2X in TLC System I (chloroform/ether, 10:3) and System II (hexane/ethyl acetate 5:2).

[c] Relative retention time in relation to the retention time of progesterone on OV 210 GC system at 35 c.c. N_2 and 230°C.

[d] Rtp of No. 4 determined by monitoring radioactivity of samples split off the detector side of the column.

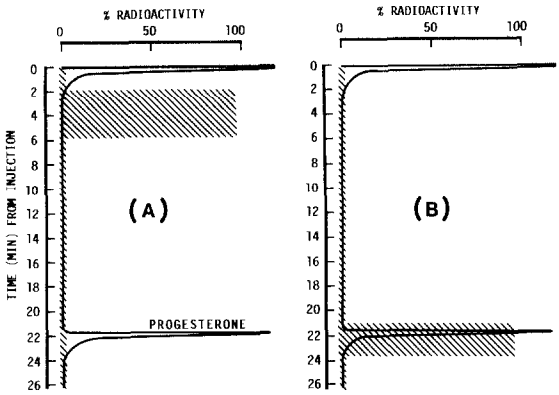

FIGURE 2. Progesterone on a GC (235°C; 35 cc/min N_2 OV-210 column (solid line) and relative retention of radioactivity (hatched area) of (A) No. 4 and of (B) No. 4 previously oxidized with CrO_3. Aliquots of No. 4 or oxidized No. 4 equivalent to about 600 dpm were injected in 2 µl methanol and samples were split off prior to the detector by means of a glass splitting device. The split portions were eluted with benzene, directly into scintillation vials and radioactivity was determined in toluene-POPOP-PPO scintillation solution by scintillation spectrometry. The figure is representative of the results of over 20 separate determinations at various conditions of temperature and N_2 flow rate.

gesterone (Figs. 2 & 3). When an aliquot of this oxidized form was crystallized with authentic progesterone, the specific radioactivity remained constant in 4 successive recrystallizations (cpm/mg of steroid were 2760, 2710, 2820 and 2800), suggesting that No. 4 contained an OH group, either in the 3 or 20 position, which had been oxidized with CrO_3 to form progesterone. Two known steroids which fit such a structure are 20α- and 20β-hydroxy-4-pregnen-3-one; however, both of these steroids behave differently than No. 4 in the TLC and GC systems, and had to be ruled out as candidates.

Evidence for the Presence of a C-3 Hydroxyl Group.

Because metabolite No. 4 could be oxidized with CrO_3 to progesterone, and since the C-20 did not appear to contain an OH, the possible presence of an OH in the C-3 position was considered by several methods. Steroids with secondary hydroxyl groups (except 11β) can be esterified

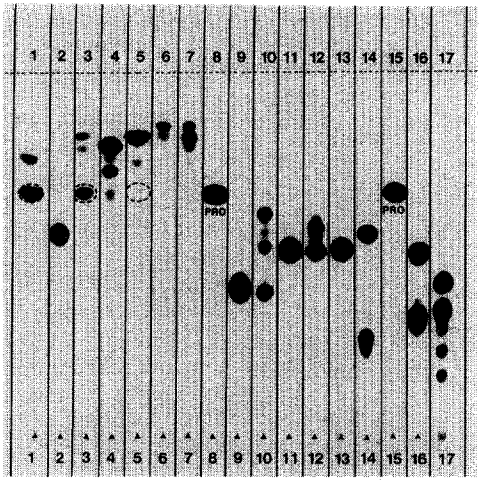

FIGURE 3. Autoradiograph showing migration of radioactivity on TLC (2X System I) of several [^{14}C] steroid standards and of No. 4 and its derivatives in the lanes shown:
(1) No. 4 oxidized with CrO_3;
(2) No. 4;
(3) No. 4 oxidized with DDQ;
(4) No. 4 acetylated;
(5) No. 4 trimethylsilyl (MSTFA);
(6) No. 4 reacted with DNPH;
(7) Progesterone (PRO) reacted with DNPH;
(8) PRO standard;
(9) 20α-DHP standard;
(10) 20α-DHP reacted with DNPH;
(11) Pregnenolone (PREG) standard;
(12) PREG reacted with DNPH;
(13) PREG oxidized with DDQ;
(14) No. 4 reacted with $NaBH_4$;
(15) PRO standard; (16) PREG reacted with $NaBH_4$; (17) 20α-DHP reacted with $NaBH_4$. Triangles indicate origin and dotted line shows position of solvent front. Dashed circles indicate position of progesterone standard detected by UV fluorescence. PRO = progesterone.

with acetic anhydride in pyridine (13). Figure 3 shows that acetylated No. 4 migrated more rapidly (higher Rf value) in the TLC system than did underivatized No. 4 or authentic progesterone. The retention time on a C-18 column in HPLC (MeOH/H_2O, 80:20; 1.5 cc/min) changed from 8.0-8.5 (No. 4) to 16.5-17.5 when No. 4 was acetylated (Fig. 4). Most secondary hydroxyl substituted steroids will also readily form trimethylsilyl ethers. The trimethylsilyl ethers show altered mobility on TLC from the parent steroid (14). Figure 3 (lane 5) shows that the silylated No. 4 moves more rapidly in TLC System I than does the underivatized No. 4 (Rfp for No. 4 = 0.81; Rfp for silylated No. 4 = 1.25).

Dichloro-5,6-dicyanobenzoquinone (DDQ) is a very mild and selective oxidant for allylic alcohols (15). When [^{14}C]-labelled No. 4 was oxi-

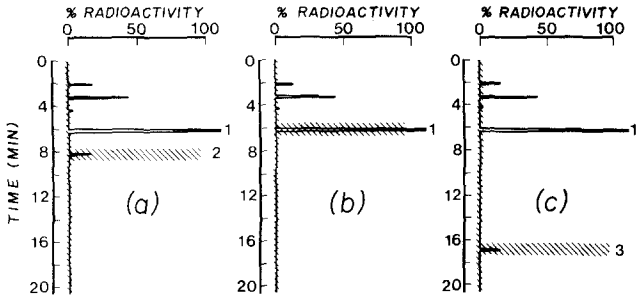

FIGURE 4. The relative retention times on HPLC [C-18 Column, methanol/water (80; 20) at 1.5 cc/min] of progesterone (peak No. 1) and radioactivity (hatched area) of (a) [^{14}C]-No. 4 (area 2), (b) oxidized [^{14}C]-No. 4; and (c) acetylated [^{14}C]-No. 4 (area 3). Aliquots of [^{14}C]-No. 4, or its derivatives were injected together with unlabelled progesterone. The eluate was monitored at 206 nm and fractions were collected every minute into scintillation vials. Ten ml of solution consisting of dioxane/naphthaline/PPO (5 g PPO and 100 g naphthalene brought to 100 ml with dioxane) was added and radioactivity was measured in a Beckman LS 255 scintillation counter. Note absorption peak under area 2 (a) and area 3 (c).

dized with DDQ (overnight, at 4°C), the product showed the same Rfp on TLC (Fig. 3) and the same Rtp on an OV-210 column in GC as authentic progesterone and a constant $^{3}H/^{14}C$ ratio = 45.6 ± 2.1). The compound, 9-borabicyclo [3.3.1]nonane (9-BBN) is a highly selective reducing agent for the facile conversion of unsaturated ketones to the allylic alcohols (16). When No. 4 was reacted with 9-BBN, there was no change in its migration rate on TLC (data not shown), further suggesting that no C-3 keto group was available. These results, taken together, indicate that No. 4 may have a 4-ene-3-ol configuration.

Evidence for a 3α-Hydroxy Configuration

The steroid oxidoreductase preparations exhibited relatively high specificities. The 3α-HSO preparation had 17.45 times more 3α-HSO activity than 3β-HSO activity with NAD and exhibited no 3β-HSO activity when NADP was used as cofactor. The 3β-oxidizing activity (~25 nmoles/

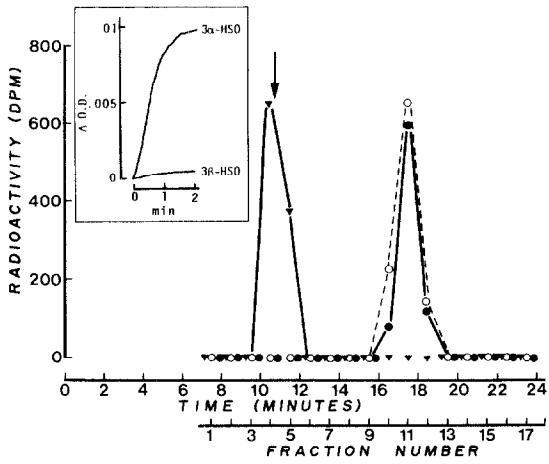

FIGURE 5. Distribution of radioactivity in HPLC fraction of No. 4 (O—O) and No. 4 reacted with either 3α-HSO (▼—▼) or 3β-HSO (●—●). About 250 ng (3500 dpm) of [^{14}C]-No. 4 was incubated with 150 µg protein of either 3α-HSO or 3β-HSO in the presence of thioNADP (50 µg) in 0.05 M phosphate buffer (pH 8.3) at 30°C. The inset shows a tracing of the changes in absorption at 395 nm (enzyme initiated reaction) as recorded by the spectrophotometer. After 2 min. the reaction mixture (1.0 ml) was extracted with 6 ml ether/chloroform (4:1). The extracts were dried down and taken up in 100 µl of methanol. Twenty µl samples were injected on the C-18 column and eluted with methanol/water (72:28) at 1.5 cc/min. Time indicates minutes from point of injection. Fractions were collected every minute into scintillation vials and radioactivity was measured by scintillation spectrometry using dioxane solution. The arrow indicates the retention time of progesterone.

min/mg) of the 3β-HSO preparation was about 162 times greater than 3α-oxidizing activity (0.11-0.15 nmoles/min/mg). No 20α-oxidizing activity could be demonstrated in either the 3α-HSO or the 3β-HSO preparations. The 20α-HSO preparation, in turn, showed no 3α- or 3β-oxidizing activities.

The reaction of about 250 ng (3500 DPM) No. 4 with either 3α-HSO, 3β-HSO or 20α-HSO preparation was followed spectrophotometrically at 395 nm with thionicotinamide adenine dinucleotide (thioNAD) or thioNADP as cofactor. (The reduced thioNAD or thioNADP has a molar extinction coefficient, at 395 nm, of 11,300, so the use of the thio compounds permits a 2-fold increase in sensitivity over reduced NAD or NADP, which

have molar extinction coefficients of 6,220 at 340 nm).

Figure 5 shows that in the presence of 3α-HSO and thioNADP, a change of 0.009 in $O.D._{395}$ occurred (equal to oxidation of about 0.79 nmoles of steroid). In the presence of 3β-HSO and thioNAD there was no reliably measurable change in $O.D._{395}$ (<0.0005 O.D. units). The reactions were allowed to proceed for 2 min, then the mixtures were extracted with ether/chloroform, the extracts were run on HPLC (C-18 column) and one ml fractions were collected and counted in a scintillation counter. Figure 5 shows that the No. 4 in reaction with 3α-HSO had been converted to a compound which showed the same Rt as progesterone while No. 4 in the presence of 3β-HSO had retained the same Rt as unreacted No. 4.

In a parallel study, ^{3}H- and ^{14}C-labelled No. 4 ($^{3}H/^{14}C$ ratio = 5.286) was incubated with either the 3α-HSO, 3β-HSO or 20α-HSO enzyme preparation, in the presence of NAD or NADP. After 2 min of incubation at room temperature, the mixtures were extracted. Each extract, along with progesterone (20 μg) and 20α-DHP (20 μg) standards, was spotted in a separate lane and run on TLC (System I). The positions of the standards were located under UV and marked on the TLC plate. Then the TLC plate was photographed in the Berthold β-spark chamber and subsequently apposed to X-ray film for 6 days. Figure 6 shows that 3α-HSO converted No. 4 to a compound which ran exactly the same as the progesterone standard (lane 1), while the result of incubating No. 4 with 3β-HSO (lanes 2 and 3) and 20α-HSO (lane 4) did not differ from an incubation in the absence of enzyme (lane 5). The radioactive spot in lane 1 was scraped and the gel eluted with ether/chloroform (4:1) and methanol. An aliquot equivalent to 18,500 DPM ^{3}H and 3,500 DPM ^{14}C was added to 26 mg progesterone and

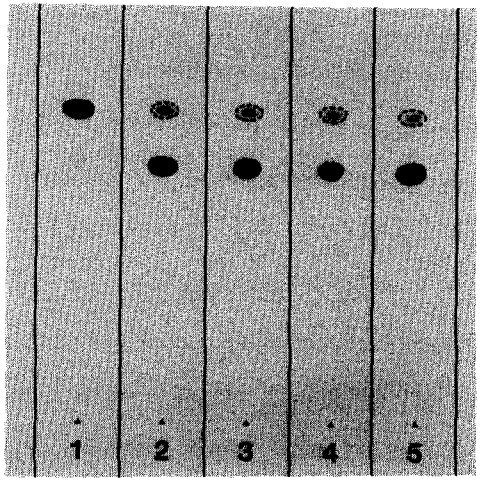

FIGURE 6. Autoradiograph showing the migration of radioactivity on TLC (2x System I) following incubation of [^{14}C]-No. 4 with 3α-HSO + NADP (lane 1), 3β-HSO + NADP (lane 2), 3β-HSO + NAD (lane 3), 20α-HSO + NADP (lane 4), and without enzyme + NADP + NAD (lane 5). The reaction mixtures consisted of No. 4 (14,000 DPM) in 30 µl methanol, 350 µl Na/K buffer (0.5 M; pH 8.3), 100 µg NADP or NAD, and 150 µg (protein) of either 3α-HSO, 3β-HSO, or 20α-HSO (lanes 1-4); for lane 5, No. 4 was incubated in buffer containing both NAD & NADP. After 2 min the reaction mixture was extracted with ether/chloroform. The extracts were concentrated under N_2 and spotted in separate lanes, together with 20 µg progesterone. The dotted areas mark the position of progesterone (visualized under UV). The plate was then exposed to X-ray film for 6 days. Note that the radioactivity in lane 1 coincides exactly with the progesterone and that only very small amounts of radioactivity fall in this region in lanes 2-5.

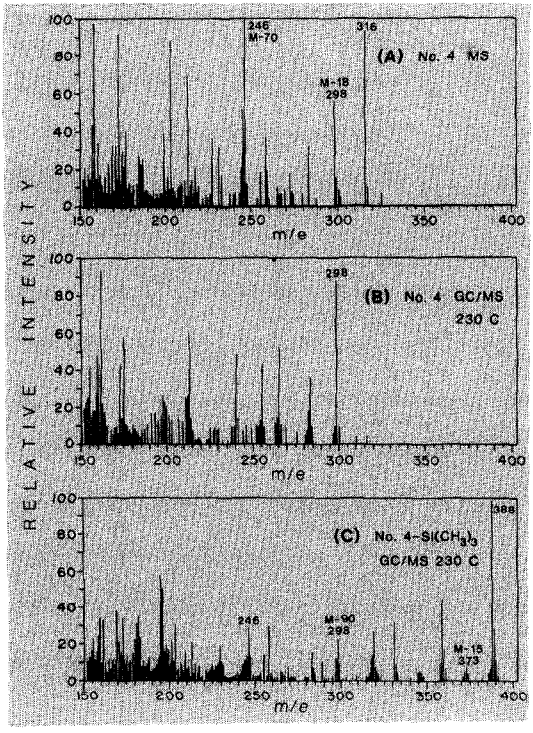

FIGURE 7. Mass spectrum at 70 eV of (A) No. 4, (B) No. 4 following GC on SE 30 column at 230°C, and (C) the trimethylsilyl (TMSi) derivative of No. 4 following GC on SE 30 column at 230°C. The mass of No. 4 (A) is 316 and the peaks at M-18 and M-70 suggest loss of H_2O and cleavage at the A ring or D ring. The M value when No. 4 is first subjected to the GC at 230°C (B) is 298, suggesting loss of water under the GC conditions. The M value of No. 4-TMSi is 388 and remains 388 even following GC; peaks also occur at M-15 (methyl from TMSi group), and at M-90 (trimethylsilanol). The peak at m/e 246 in (C) may be due to A-ring cleavage to yield an ion containing the TMSi ether group.

the mixture crystallized from acetone/pentane. Specific activity of crystals from 4 successive crystallizations varied by less than 5% from the calculated activity (activity in DPM/mg: calculated, 846; Crystals I, 859; Crystals II, 870; Crystals III, 862; Crystals IV, 860) and the $^{3}H/^{14}C$ ratio remained at 5.286 suggesting that No. 4 had been converted to progesterone by 3α-HSO.

Evidence for the Presence of a Carbonyl

Several reactions indicate that No. 4 contains a carbonyl group. A common derivatization reagent for carbonyls is 2,4-dinitrophenylhydrazine (17). The dinitrophenylhydrazone of No. 4 had a migration rate on TLC which differed from that of underivatized No. 4 and from the DNPH derivatives of progesterone, 20α-DHP and pregnenolone (Fig. 3). Reduction of No. 4 with $NaBH_4$ produced a more polar product than No. 4 (Fig. 3) suggesting the formation of a dihydroxy compound.

Mass Spectrometric Confirmation of Structures

On the basis of the above chemical and enzymatic observations it was proposed that No. 4 metabolite was 3α-hydroxy-4-pregnen-20-one (3α-dihydroprogesterone; 3α-DHP) and therefore had a molecular weight of 316. Samples of No. 4 applied to mass spectrometric analysis by direct probe showed a mass of 316 (Fig. 7A). However, when samples were first run on the GC at 230°C, the spectrum indicated an upper mass of 298 (Fig. 7B), suggesting that an H_2O molecule had been lost during gas chromatography. The lability of the compound under GC conditions might help to explain why it had not been possible to obtain a distinct peak when run on either an OV 210, SE30 or OV17 column, at 220-240°C. To determine if the molecule was relatively unstable during GC conditions at the 3α-hydroxy

site, samples were trimethylsilylated and subjected to GC/MS analysis. Trimethylsilyl ethers of secondary hydroxyl substituted steroids generally have excellent properties for GLC work as the ethers are thermally stable and the bulky group provides steric shielding for the oxygen atom (14). The mass of trimethylsilylated No. 4 as determined by GC/MS, was 388 (Fig. 7C) which is identical to the calculated mass of trimethylsilylated 3α-hydroxy-4-pregnen-20-one.

The Structure of Sertoli Cell Metabolite No. 4 (3α-DHP)

It is concluded that the structure of Sertoli cell metabolite No. 4 is 3α-hydroxy-4-pregnen-20-one (Fig. 8).

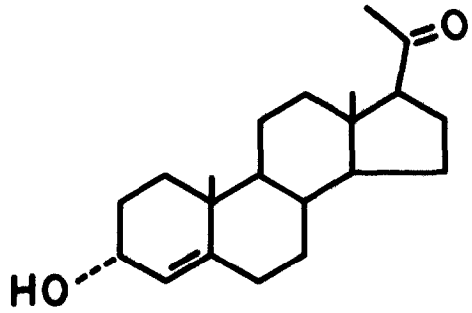

FIGURE 8. The structure of metabolite No. 4 (3α-hydroxy-4-pregnen-20-one; 3α-DHP).

Other Characteristics of 3α-DHP

In contrast to many steroids which are stable in daylight, at room temperature and even in GC conditions at 220-240°C, 3α-DHP appears to be a relatively unstable molecule. So far it has not been possible to obtain a good single peak of underivatized 3α-DHP on the GC and it appears that H_2O is lost (Fig. 7B) at high temperature. Reacting 3α-DHP with

heptafluorobutyric anhydride at 70°C did not produce a derivative which could be detected on the GC equipped with an electron capture detector, and it is assumed that the high temperature resulted in dehydration or oxidation. It has long been recognized that a steroid with an allylic alcohol is rather reactive and easily dehydrated to a diene system (13) or oxidized to a ketone. 3α-DHP appears to be readily oxidized. On several occasins, when 3α-DHP was maintained under N_2, at 45°C, for about 10 min following evaporation of the benzene or methanol solvent, HPLC and crystallization data showed that a large part of the sample had been oxidized to progesterone. Similarly, exposure of dry samples to fluorescent light appeared to oxidize the 3α-DHP. On the other hand, storage at 4°C in HPLC grade methanol or in benzene/ethanol (9:1) appears to keep a large percentage of 3α-DHP intact for as long as 18 months.

Biological Significance of 3α-DHP from Sertoli Cells

The allylic 3α-DHP has been identified as a metabolite of uterine (18) and fetal blood cell (19) preparations but its possible biological functions are unknown. However, from our Sertoli cell studies, two types of information suggest that 3α-DHP is more than a waste metabolite of progesterone. First, the amount of 3α-DHP produced by Sertoli cells varies with the age of the animals (Table 2); production on a ng/mg Sertoli cell protein basis increases about 4 fold between 6 and 10 days of age (158.5 to 591.5 ng/mg), remains relatively high until 17 days of age and then continues to be produced at lower levels at 25 days and thereafter (Table 2). The peak in 3α-DHP production coincides with the peak in the production, by Sertoli cells, of a number of other steroids (6), with the peak in FSH binding (20), and with the cellular events marking

Table 2. In vitro production of No. 4 (in ng/mg protein ± S.E.) by Sertoli cells from rats of different ages

Incubation Time	Age (Days)					
	6	10	17	25	32	65
6 hours	159.0 (± 2.2)	386.4 (± 10.7)	275.9 (± 14.7)	117.9 (± 1.1)	38.8 (± 2.2)	11.3 (± 1.1)
20 hours	158.5 (± 1.5)	591.5 (± 32.7)	467.9 (± 30.5)	148.1 (± 9.5)	150.3 (± 32.6)	137.6 (± 27.8)

Sertoli cells were isolated from rats at the ages indicated and were cultured as described in Materials and Methods. Approximately 10^6 cells were cultured per petri dish and then were incubated in the presence of [^{14}C]-progesterone (0.65 µCi/11.56 nmoles) for either 6 or 20 hours. No. 4 was located on the TLC plates by autoradiography; the gels were eluted and No. 4 was quantitated by scintillation spectrometry. Recovery of No. 4 was not monitored directly; instead, recovery calculations are based on recovery of ^{3}H-labelled progesterone and testosterone. The number of replicate cultures for each age and incubation tissue was four or five. Protein was determined by the method of (22).

the initiation of meiosis in the rat (21). The second piece of information results from binding studies. [^{3}H]-labelled 3α-DHP binds to testicular homogenate and the binding appears to be specific: [^{3}H]-3α-DHP can be displaced by unlabelled 3α-DHP but not by 1000 fold excess of progesterone (unpublished observations). At present we do not know which cells possess the receptor molecules nor have we information about the kinetics of binding. However, the evidence of specific binding might suggest a biological function. The observation that 3α-DHP is produced in Sertoli cells but not in Leydig cells, suggests that its production does not occur in all steroidogenic tissues and that it is not merely a product of isolation and purification methodology. Recently, we have also found 3α-DHP when homogenate of quail (Coturnix coturnix japonica) testes was incubated with [^{14}C]-progesterone; as in the rat, 3α-DHP pro-

duction shows an age-related variation (unpublished results). As the peak in production of 3α-DHP occurs at the onset of male gametogenesis, studies are in progress to determine the possible role of this newly identified Sertoli cell steroid in testicular gametogenesis and endocrinology.

Synthesis of 3α-DHP

Numerous attempts have been made to synthesize 3α-DHP. Reaction with the 3α-HSO enzyme preparation indicated rapid oxidation of 3α-DHP in the presence of NADP (Figs. 5 and 6). Consequently, attempts were made to reverse the reaction by incubating progesterone with 3α-HSO in the presence of reduced NADP and at several pH conditions. So far, it has not been possible to synthesize 3α-DHP by these enzymatic procedures. It may be that in the Sertoli cells, a particular enzyme exists for the 3α reduction of progesterone, or that another cofactor or other molecule is involved in the reaction, or that specific compartmentalization of substrate, cofactor, and/or enzyme occurs in Sertoli cells. If it were not so, 3α-DHP production might be expected also in Leydig cells, since they contain abundant 3α-HSO activity (9).

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