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ANALYSIS FOR THE 5α-STEROID REDUCTASE IN THE BOVINE OVARY WITH [^{14}C]-PROGESTERONE AND [^{14}C]-TESTOSTERONE

THOMAS H. WISE and M. J. FIELDS
Animal Science Department, Livestock Pavilion, University of Florida, Gainesville, Florida 32611, U.S.A.

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SUMMARY

A series of experiments were organized to elucidate the presence and activity of the 5ξ-steroid reductase enzyme in the bovine ovary by incorporating various hormonal stimuli [follicle stimulating hormone (FSH), luteinizing hormone (LH) or prolactin], substrates ([^{14}C]-4-pregnene-3,20-dione, progesterone or [^{14}C]-17β-hydroxy-4-androsten-3-one, testosterone), ovarian tissues (follicular, luteal, corpora albicantia or stroma) and time intervals (5, 10, 20, 45 and 120 min). Metabolites monitored for evidence of reductase activity were 5α-pregnane-3,20-dione (5α-dihydroprogesterone), 5α-androstane-3,17-dione (5α-androstanedione) and 17β-hydroxy-5α-androstan-3-one (5α-dihydrotestosterone). Initial isolation and tentative identification of metabolites was performed using Sephadex LH-20 chromatography followed by acetylation and recrystallization to constant specific activity for final determination. Preliminary data from column chromatography revealed occasional evidence of low conversions of [^{14}C]-progesterone to [^{14}C]-5α-dihydroprogesterone; however, subsequent crystallizations proved this to be a contaminant. Analysis for [^{14}C]-5β-pregnane-3,20-dione (5β-dihydroprogesterone) also proved negative. Conversion of [^{14}C]-testosterone to [^{14}C]-4-androstene-3,17-dione (androstenedione) and more polar metabolites was noted; however, no conversion to [^{14}C]-5α-dihydrotestosterone or [^{14}C]-5α-androstanedione was detected. The 5α- and 5β-steroid reductase enzymes within the bovine ovary were found to be either extremely inactive or absent under these *in vitro* conditions based on the lack of conversion of [^{14}C]-progesterone or [^{14}C]-testosterone to reduced metabolites, i.e. 5α-dihydroprogesterone, 5α-androstanedione or 5α-dihydrotestosterone.

INTRODUCTION

Recently, evidence has accumulated to substantiate the concept that the 5α- and 5β-reduced steroids are playing an active role in feedback mechanisms [1–5], modulation of steroid metabolism [6–9] and physiological responses [10–13] in the female and male. Production of 5α-reduced steroids has been attributed to mammary [14–16], adrenal [9, 17, 18], gonadal [19–22] and higher brain center tissues [23–27]. In view of the proposed importance of 5α-reduced steroids, the primary objective was to establish presence, ovarian tissue origin and gonadotropin effect on the 5α-reductase enzyme in the bovine ovary.

A secondary objective was to provide more specific information on the presence of the 5α-reduced steroids which interfere with immunological assays that are presently utilized for endocrine interpretations of reproductive steroids in the bovine. Such a problem was noted by Holtan *et al.*[28] when unknown steroids interfered with immunological assays for plasma progesterone in the mare. These were later identified as 5α-reduced progestins [29]. In the pregnant mare Atkins *et al.*[30] found plasma levels of 5α-dihydroprogesterone similar to those of progesterone, while Dunn[31] noted plasma levels of 21-hydroxy-5α-pregnane-3,20-dione were considerably higher than the nonreduced counterpart, 21-hydroxy-4-pregnene-3,20-dione. Analysis of blood serum in pregnant and non-pregnant cattle has failed to indicate a measurable presence of 5α-reduced progestins by radioimmunoassays [32, 33] or gas–liquid chromatography and mass spectrometry [29].

MATERIALS AND METHODS

Solvents. Solvents were distilled over $CaCl_2$ with the exception of ether which was distilled over metallic sodium. Hexane and cyclohexane were washed with sulfuric acid and water prior to distillation over NaOH pellets.

Radioactive steroids. Substrates ([4-^{14}C]-progesterone, 55.7 mCi/mmol and [4-^{14}C]-testosterone, 57.5 mCi/mmol) and steroids used for recovery purposes ([1,2-^{3}H]-dihydroprogesterone, 48 Ci/mmol, [1,2-^{3}H]-androstenedione, 40 Ci/mmol, [1,2-^{3}H]-dihydrotestosterone, 44 Ci/mmol, [1,2-^{3}H]-testosterone, 40 Ci/mmol and [1,2-^{3}H]-progesterone, 48 Ci/mmol) were supplied by New England Nuclear and chromatographed on Sephadex LH-20 columns prior to use.

Tissues and incubation. Experiment I was a pilot study in which the ovaries were excised from a cow on day 18 of the estrous cycle following treatment on day 16 with 2000 IU pregnant mare serum gonadotropin (PMSG; Organon) for maximum ovarian

stimulation and follicular growth. Ovarian slices 5 mm thick and weighing approximately 50 mg were incubated in duplicate with 5 ml Krebs–Ringer bicarbonate buffer (pH 7.2) with 75 μmol of NADH, 75 μmol NADPH (Sigma Chemicals), 0.1% glucose, 1.2 μCi of [4-^{14}C]-progesterone or 1.33 μCi of [4-^{14}C]-testosterone. Stimulatory gonadotropins utilized were (NIH-LH-B9, NIH-FSH-B1 and NIH-Prolactin-B4 (100 μg/flask). Ovarian slices were incubated in duplicate for either 5, 10, 20 or 45 min at 37°C with radioactive substrate in conjunction with the gonadotropins (Table 1). Samples were incubated in a Dubnoff metabolic shaker (60 cycles/min) with an atmosphere of oxygen–carbon dioxide (95:5). Homogenized ovarian tissue and oviduct were also included to test activity and presence of the reductase enzyme (Table 1).

Experiment II involved three Angus cows in which estrus had been synchronized with two injections of prostaglandin $F_{2\alpha}$ (32.5 mg; The Upjohn Co.) at 12 day intervals. A 2000 IU injection of PMSG was given 24 hr prior to the second prostaglandin injection. This treatment scheme placed all animals near the same stage of follicular development as in Experiment I. Two days after the second prostaglandin injection the ovaries were excised. The follicles were then dissected out, trimmed free of connective tissue, minced and 50 mg of minced tissue added to each incubation flask. The protocol for incubation in Experiment II was similar to that for Experiment I, except that no cofactors were utilized and only 3 ml of buffer per incubation flask were utilized to increase a steroid gradient into the tissue. An increased time interval was added (120 min) to observe possible increases in 5α-reductase activity during extended incubation periods (Table 1).

In Experiment III, three Angus cows were utilized in a pretreatment protocol similar to Experiment II. Ovaries were excised at varying stages of the estrous cycle to provide corpora lutea, corpora albicantia, ovulatory follicles and stromal tissues in an attempt to identify possible 5α-reductase tissue specificity in the bovine ovary. Intact follicles (less follicular fluid) were trimmed from the ovary and incubated in 3 ml buffer. All other tissues were removed from the ovary, minced and 100 mg of minced tissue added to each incubation flask containing 3 ml of buffer. Other procedures were similar to those used in Experiment II (Table 1).

In conjunction with bovine ovarian tissues, mouse testes, ovaries and livers were incubated in each experiment to monitor the reliability of the incubation system and to aid in the identification, purification and recrystallization procedures for the 5α-reduced steroids.

Extraction. Prior to homogenization, tritiated steroids (5α-dihydroprogesterone, progesterone, androstenedione, 5α-dihydrotestosterone and testosterone) were added to each incubate to monitor procedural losses and to serve as markers for column chromatography. Incubated tissues were homogenized (Ten Broeck) and percolated twice for 6–8 hr in 50 ml of methanol. The supernant was recovered after centrifugation (40,000 *g*) and the volume subsequently reduced to 2 ml under nitrogen gas. 2 ml of distilled water were added to the dried down extract and subsequently extracted twice with 35 ml of ether.

Column chromatography. Tentative identification of [^{14}C]-5α-dihydroprogesterone was perfomed on Sephadex LH-20 columns (1 × 17 cm: Hexane–Benzene–Methanol: 90:5:5, v/v) and for [^{14}C]-testosterone metabolites (1 × 19 cm: Cyclohexane–Benzene–

Table 1. Experimental protocol for incubating [^{14}C]-progesterone (P) and [^{14}C]-testosterone (T) with the bovine ovary

Experiment I[a]	Experiment II[a]	Experiment III[a]
Ovarian slices	Follicular minces	Stromal minces
P+ Control	P+ Control	P+ Control
P+ LH	P+ LH	P+ LH
P+ FSH	P+ FSH	P+ FSH
P+ Prolactin	P+ Prolactin	P+ Prolactin
T+ Control	T+ Control	T+ Control
T+ LH	T+ LH	T+ LH
T+ FSH	T+ FSH	
Ovarian Homogenate		Corpora Luteal minces
P+ Control		P+ Control
T+ Control		P+ LH
Oviduct		Corpora Albicantical minces
P+ Control		P+ Control
T+ Control		T+ Control
		Intact Follicles
		P+ Control
		T+ Control

[a] Experiment I was incubated for 5, 10, 20 and 45 min and Experiment II and III for 5, 10, 20, 45 and 120 min. Control was incubated with no FSH, LH or Prolactin.

Methanol: 90:25:5, v/v). Eluates were collected in 1 ml fractions, from which 100 μl aliquots were removed for liquid scintillation counting to correlate ^{14}C elution profiles with known tritiated steroid markers added prior to extraction (Figs. 1 and 2).

Crystallizations. Non-radioactive steroids (Steraloids Inc.) were recrystallized prior to use. Incubated samples revealing possible reduced ^{14}C metabolites from column chromatography were subjected to recrystallization to constant specific activity for final analysis and identification.

RESULTS

Chromatographic results from Experiment I revealed no conversion of [^{14}C]-testosterone to [^{14}C]-5α-dihydrotestosterone during any of the time periods or treatments. A peak was noted matching the androstenedione chromatographic marker prior to and following acetylation (acetic anhydride: pyridine, 1:1, for 60 min). Successive crystallizations to constant specific activity established this metabolite as androstenedione (Table 2). Also, to be noted from the chromatographs (Fig. 2), is the lack of any 5α-reduced androstenedione (5α-androstanedione).

Preliminary results from the chromatography of [^{14}C]-progesterone incubates from Experiment I revealed a small amount of activity in peaks matching 5α-dihydroprogesterone markers during the extended incubation times of 20 and 45 min in the LH, FSH and prolactin stimulated slices (Fig. 1). Most samples revealed no conversion to 5α-dihydroprogesterone (Fig. 1a). The small tentative conversion at extended times suggested an inactive enzyme, or a shortage of enzymatically active tissue, since the ovarian slices were primarily stromal tissue.

In an attempt to localize and concentrate the enzymatic activity, an incubation of follicular minces was pursued. Results from Experiment II were similar to those of Experiment I in that no conversion of [^{14}C]-testosterone to [^{14}C]-5α-dihydrotestosterone was detected by column chromatography under any of the experimental conditions. Androstenedione was detected and identified as a metabolite of [^{14}C]-testosterone (Table 2). In addition, a metabolite more polar than testosterone and less polar than estrone was observed in only the LH stimulated incubates (Fig. 2c). Chromatographic analysis has indicated that this unknown is not likely to be 3β-hydroxy 1,3,5,(10)-estratrien-17-one (estrone), 5α-androstane-3β, 17β-diol or 17α-hydroxy-4-androsten-3-one (epitestosterone) (Fig. 3). Chromatographs of the [^{14}C]-progesterone in Experiment II were similar to those found in Experiment I, i.e. tentative low activity in the 5α-dihydroprogesterone fractions from samples incubated for the longer times (Fig. 1b, c).

A further analysis of tissue specificity was pursued in Experiment III utilizing intact follicles, corpora lutea minces, corpora albicantia minces and stromal minces. Similar to Experiment I and II, column chromatography indicated no detectable conversion of [^{14}C]-testosterone to [^{14}C]-5α-dihydrotestosterone (Fig. 2d) and only tentative evidence for conversion of [^{14}C]-progesterone to [^{14}C]-5α-dihydroprogesterone (Fig. 1d).

Table 2. Recrystallization analyses for androstenedione and 5α-androstanedione following chromatographic separation

Recrystallization No.	0 ^3H ^{14}C	1 ^3H ^{14}C	2 ^3H ^{14}C	3 ^3H ^{14}C
	Androstenedione-$^3H/^{14}C$ in Androstenedione Crystals (dpm/mg)			
Experiment I—ovarian slices				
Control — 5 min	1060–367	934–316	955–343	918–340
FSH — 5 min	1250–1088	1176–1032	1184–1062	1166–1050
FSH — 20 min	902–932	1144–1211	1189–1195	1176–1161
LH — 20 min	1028–342	1039–350	1018–365	1031–348
LH —120 min	1400–1433	1134–1260	1455–1486	1429–1405
Experiment II—follicular minces				
FSH — 5 min	289–286	286–317	297–305	288–309
FSH — 10 min	634–541	576–600	607–551	599–579
LH — 10 min	957–873	947–877	910–840	905–873
FSH — 45 min	726–755	671–723	697–706	680–730
Control —120 min	1126–1666	1029–1177	1068–1116	1047–1138
Experiments II and III	[a]Androstenedione-$^3H/^{14}C$ in 5α-androstanedione crystals (dpm/mg)			
LH — 5 min	371–315	71–55	42–25	8–7
Control — 10 min	226–194	39–34	13–11	2–2
FSH — 20 min	121–101	34–31	31–18	3–3
Control — 45 min	139–112	18–15	10–4	4–3
LH — 45 min	126–87	45–43	16–12	3–3
FSH —120 min	345–317	84–81	39–26	12–7
Control —120 min	163–140	66–46	37–25	5–8

[a] Determination that androstenedione-$^3H/^{14}C$ was not 5α-androstanedione.

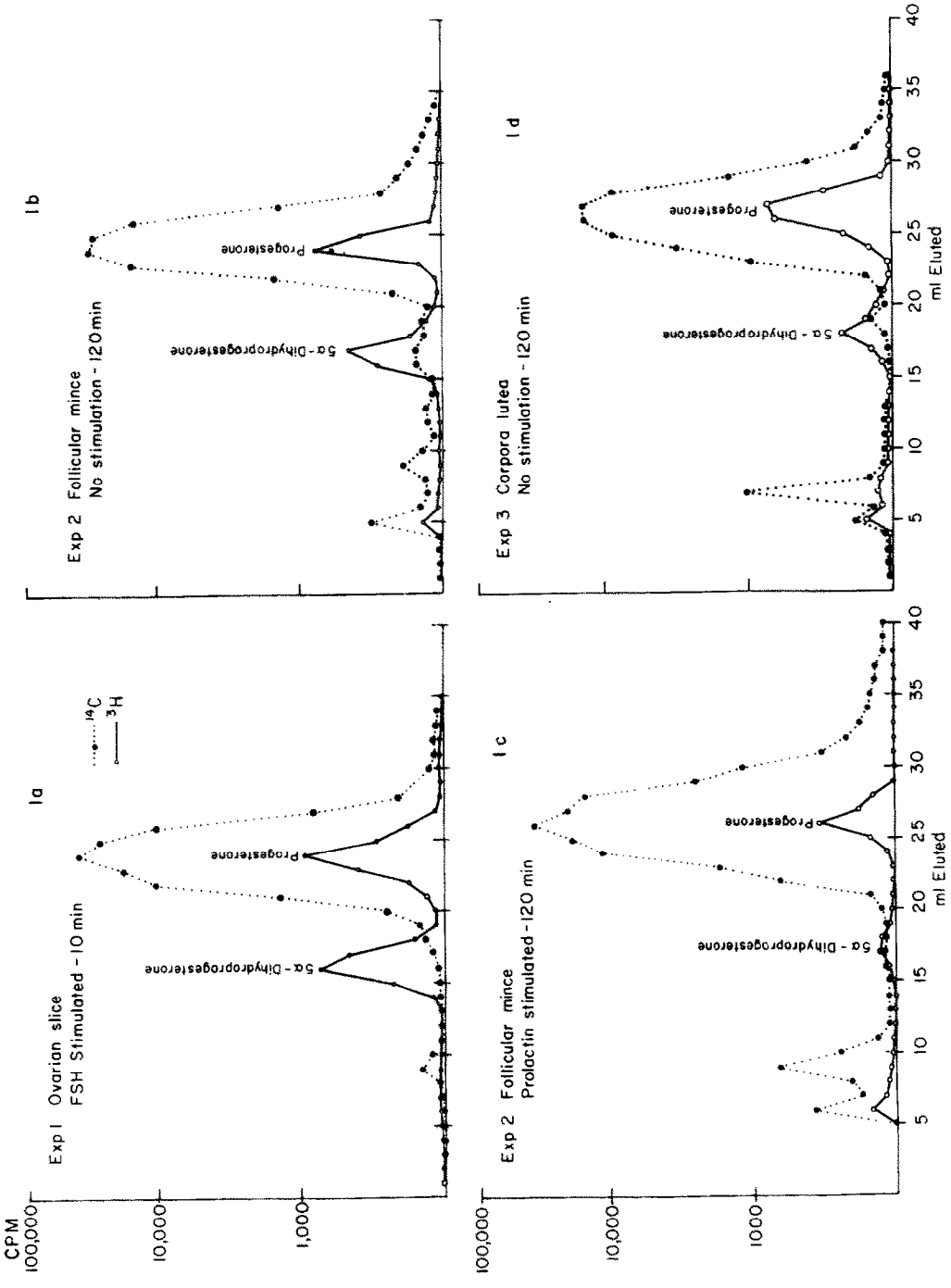

Fig. 1. Representative Sephadex LH-20 chromatographs (Hexane–Benzene–Methanol, 90:5:5 by vol) from Experiment I. II and III of [^{14}C]-progesterone-metabolites.

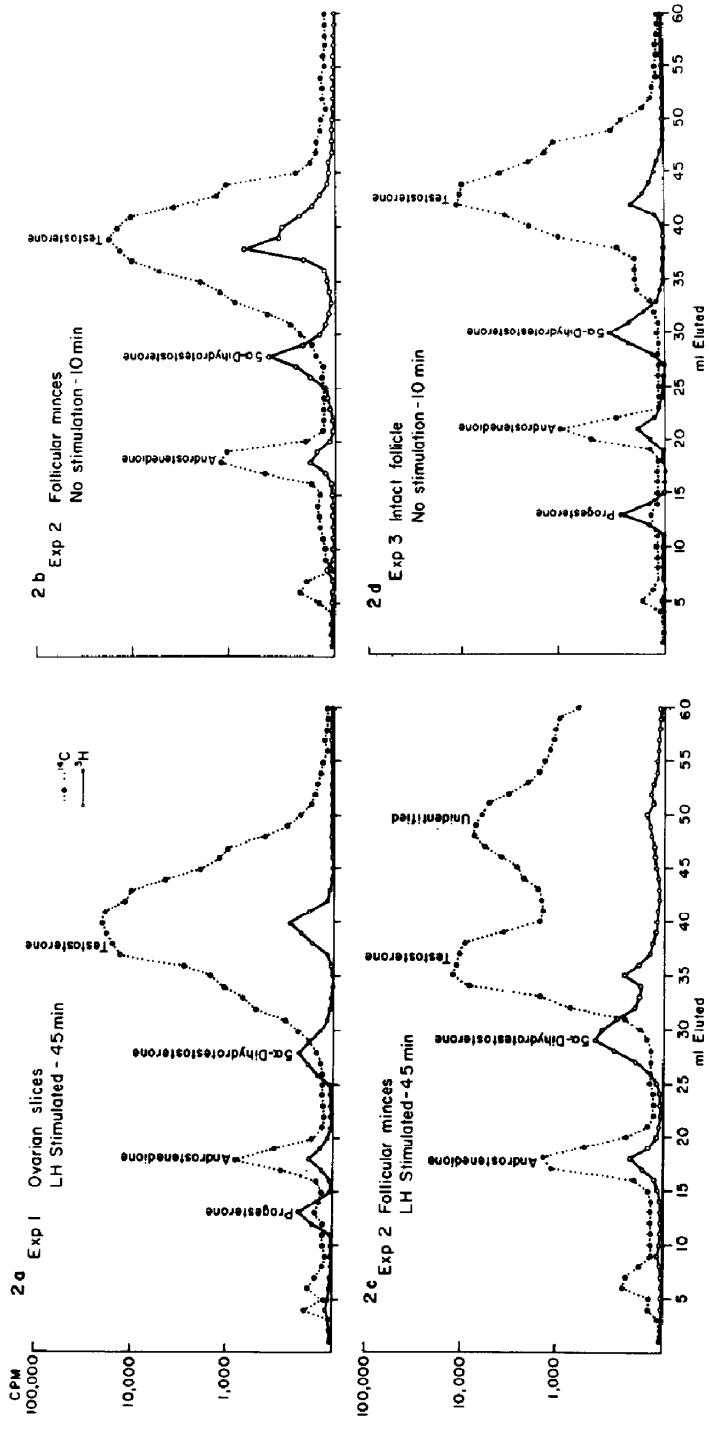

Fig. 2. Representative Sephadex LH-20 chromatographs (Cyclohexane–Benzene–Methanol, 90:25:5 by vol) of [^{14}C]-testosterone metabolites from Experiments I, II and III revealing no conversion to [^{14}C]-5α-dihydrotestosterone.

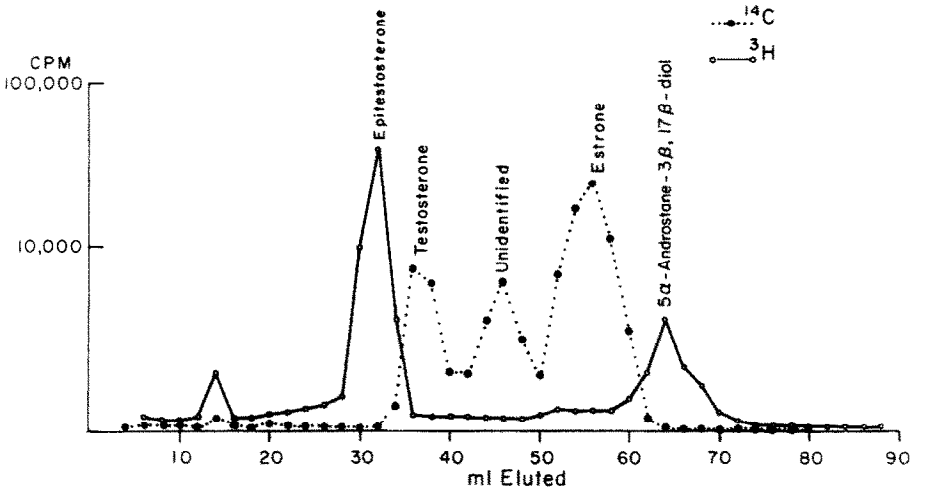

Fig. 3. Sephadex LH-20 chromatographic analysis (1 × 16 cm column, Cyclohexane–Benzene–Methanol. 90:25:5 by vol) of unknown [^{14}C]-testosterone metabolite from LH stimulated follicular minces in Experiment II.

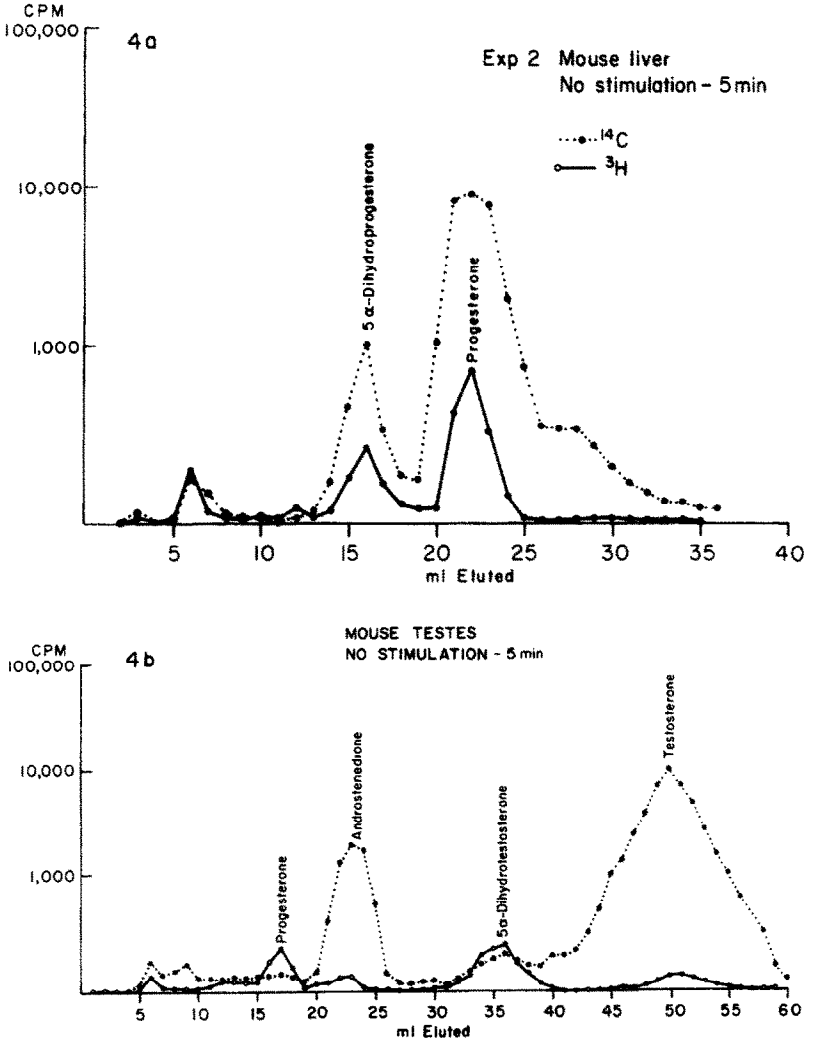

Fig. 4. Representative Sephadex LH-20 chromatographs (4a. Hexane–Benzene-Methanol. 90:5:5 by vol. 4b. Cyclohexane–Benzene-Methanol. 90:25:5 by vol) of mouse control tissues incubated with [^{14}C]-progesterone (4a) and [^{14}C]-testosterone (4b) demonstrating conversion to reduced metabolites.

Table 3. Recrystallization analyses for 5α-dihydroprogesterone following chromatographic separation

Recrystallization No.	0 ^3H ^{14}C	1 ^3H ^{14}C	2 ^3H ^{14}C	3 ^3H ^{14}C
	5α-dihydroprogesterone-$^3H/^{14}C$ in 5α-dihydroprogesterone crystals (dpm/mg)			
Experiment I—ovarian slices				
FSH —20 min	1913–101	1634–21	1690–15	1718–8
LH —20 min	2306–207	1828–41	1846–28	1865–17
Prolactin —45 min	4447–134	4043–56	3418–20	3325–16
Experiment II—follicular minces				
FSH — 5 min	1615–81	1325–20	1340–13	1331–10
LH — 45 min	2325–86	2022–38	1928–18	2040–14
Control —120 min	2306–206	1828–41	1848–27	1856–24
Experiment III				
Stroma + Prolactin —120 min	625–61	512–29	506–23	516–11
Corpora Lutea —120 min	2490–85	2387–34	2368–24	2250–18
Corpora Lutea + LH —120 min	2059–100	1800–50	1772–21	1790–14
Mouse Tissues				
Liver —15 min	1781–514	1525–380	1516–388	1459–373
Testis —10 min	866–212	842–208	853–219	849–212

A system of control tissues consisting of mouse ovaries, testes and livers were incubated throughout these series of experiments. The conversion of [^{14}C]-progesterone to [^{14}C]-5α-dihydroprogesterone and [^{14}C]-testosterone to [^{14}C]-5α-dihydrotestosterone was detectable after only 5 min of incubation and indicated that the incubation system was viable (Fig. 4a, b).

Samples tentatively identified by column chromatography as [^{14}C]-5α-dihydroprogesterone and [^{14}C]-5α-dihydrotestosterone were subjected to recrystallization to constant specific activity, for final identification. As shown in Table 3, samples suspected to be [^{14}C]-5α-dihydroprogesterone did not recrystallize to a constant specific activity although the [^3H]-5α-dihydroprogesterone added for recovery and chromatographic analysis did recrystallize to a constant specific activity. The mouse control tissue (testis, liver) [^{14}C]-5α-dihydroprogesterone did recrystallize to a constant specific activity (Table 3).

An analysis for possible 5β-dihydroprogesterone was carried out on a number of samples, and Table 4 demonstrates that 5α-dihydroprogesterone can be separated from its 5β-epimer utilizing [^3H]-5α-dihydroprogesterone in 5b-dihydroprogesterone crystals in a series of recrystallizations. This separation was accomplished with a very slow growth of crystals over 10–15 days as seen in recrystallizations 3, 4 and 5, whereas routine recrystallizations 1 and 2, for 48 h, maintained activity. Table 4 further shows a number of routine recrystallizations in which the [^3H]-5α-dihydroprogesterone remained unchanged while the ^{14}C contaminant rapidly decreased in specific activity. The ^{14}C found in the dihydroprogesterone fraction did not recrystallize to a constant specific activity with nonradioactive 5β-dihydroprogesterone.

Table 2 summarizes the recrystallization results of isolated [^{14}C]-androstenedione following acetylation. In addition, Table 2 summarizes the results of recrystallizations of the [^{14}C]-androstenedione in 5α-androstanedione crystals. The data reveal no 5α-reduced androstenedione produced in conjunction with [^{14}C]-androstenedione.

Recrystallization showed conversion of [^{14}C]-tes-

Table 4. Recrystallization analyses for 5β-dihydroprogesterone following chromatographic separation

Recrystallization No.	0 ^3H ^{14}C	1 ^3H ^{14}C	2 ^3H ^{14}C	3 ^3H ^{14}C	4 ^3H ^{14}C	5 ^3H ^{14}C
	5α-dihydroprogesterone-^3H in 5β-dihydroprogesterone crystals (dpm/mg)					
Separation of α/β isomers[a]	2420	1979	2082	1744	897	305
	5α-dihydroprogesterone-$^3H/^{14}C$ in 5β-dihydroprogesterone crystals (dpm/mg)					
Experiment III[b]						
Follicle 1.5 × 2 mm, 10 min	1097–65	1103–60	1221–47	1215–9		
Follicle 0.5 × 0.6 mm, 20 min	2209–152	1962–76	2209–52	2231–45		
Follicle 2 × 2 mm, 10 min	443–61	403–30	318–17	306–11		

[a] Crystallization No. 1 and 2 for 48 h; crystallization No. 3, 4 and 5 for 10–15 days.
[b] Crystallization for 24–72 h.

tosterone to [^{14}C]-5α-dihydrotestosterone by control tissues from mouse testes, ovaries and livers. However, bovine ovarian tissues produced no detectable conversion of testosterone to 5α-dihydrotestosterone.

DISCUSSION

In vitro incubations have demonstrated that the presence of the 5α-reductase enzyme is usually accompanied by an active and quick conversion of substrate [19]. In the bovine ovary, the 5α-reductase enzyme for converting progesterone to 5α-dihydroprogesterone or testosterone to 5α-dihydrotestosterone may be either nonexistent, inactive in the system used or reactive, but synthesizing metabolites more polar than this analysis tested. No reduced androstenedione was noted in incubates that metabolized testosterone to androstenedione. In an attempt to stimulate the action of these enzymes and substantiate their existence, tissues were incubated with pituitary gonadotropins and prolactin since previous work indicated that they may have a stimulatory effect on the reductase system [9, 19, 22, 25].

It has been shown that 3β-hydroxysteroid dehydrogenase, in the bovine ovary, is substrate specific and that more than one isoenzyme exist [34, 35]. Bovine spermatozoa have also been shown to reduce androstenedione to 5α-androstanedione, but not testosterone to 5α-dihydrotestosterone, suggesting substrate specificity for the reductase enzyme [36].

Axelson *et al.*[37] demonstrated the presence of 5α-reduced pregnandiols in bovine corpora lutea, but found no 5α-dihydroprogesterone. Holtan *et al.*[29] found no pregnanes in plasma of pregnant cows. Cardinali *et al.*[38] found the bovine pineal gland incapable of metabolizing testosterone to reduced steroids, and Kanchev *et al.*[39] found no dihydrotestosterone in the bovine peripheral plasma during the estrous cycle.

In some experiments that analyzed for the 5α-reduced steroids in farm animals, the levels were as high or higher than the parent steroid and cross-reacted with the antisera utilized in quantitation of the endocrine relationships [28, 30, 31]. Possible interference and incorrect interpretations of radioimmunoassay results from the presence of 5α-reduced steroids could be eliminated by extensive chromatography prior to quantitation by radioimmunoassay, but this considerably increases the time and expense involved for analysis. The levels and ease of conversion of steroids to the 5α-reduced form in many tissues suggest a dominant role in the female. It is important to isolate and identify the 5α-reduced steroids in order to maintain a correct interpretation of the role of circulating reproductive steroids.

In conclusion, the 5α- and 5β-reductase enzyme within the bovine ovary was found to be unreactive in metabolizing [^{14}C]-progesterone to [^{14}C]-5α-dihydroprogesterone or [^{14}C]-testosterone to [^{14}C]-5α-dihydrotestosterone under these *in vitro* conditions. However, endocrine tissues from mice, incubated under the same conditions, were active in reducing these substrates. In the rat, ovarian 5α-dihydrotestosterone may antagonize the effect of FSH on developing follicles and, depending on the metabolism of testosterone (to estrogen or 5α-dihydrotestosterone), follicular growth may be stimulated or inhibited [1, 6]. Lack of evidence for the 5α-reductase enzyme in the bovine ovary points to an alternate regulatory mechanism for follicular growth and selection of the follicle destined to develop to maturity. Lack of detectable 5α- and 5β-reductase activity in the bovine ovary may also provide for a more specific, reliable and simple analysis of reproductive steroids in the bovine female.

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