METABOLISM AND CONJUGATION OF [4-^{14}C]PROGESTERONE BY BOVINE LIVER AND ADIPOSE TISSUES, *IN VITRO*

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ABSTRACT

The ability of bovine liver and fat to metabolize progesterone and also to form glucuronide conjugates with these progestins *in vitro* was investigated. Tissue supernatants were incubated with [4-^{14}C] progesterone, UDP-glucuronic acid, and a NADPH generating system for 5 hr, at 37°C. Steroids were identified by thin-layer chromatography, high performance liquid chromatography, and recrystallization to a constant specific activity. The total original radioactivity which could not be removed by exhaustive ether extraction (presumptive conjugates) was 44.7 ± 14.2% in liver, 5.0 ± 3.6% in subcutaneous fat, and 3.7 ± 2.2% in kidney fat samples. Progestins identified in liver samples include 5β-pregnane-3α,20α-diol (free and conjugate), 5β-pregnane-3α,20β-diol (free and conjugate), 3α-hydroxy-5β-pregnan-20-one (free and conjugate), 3β-hydroxy-5β-pregnan-20-one (free), 5β-pregnane-3,20-dione (free), and progesterone (conjugate). Progestins identified in both the free and conjugate fractions of subcutaneous fat and kidney fat samples include progesterone, 3α-hydroxy-5β-pregnan-20-one, 20β-hydroxy-4-pregnen-3-one, and 20α-hydroxy-4-pregnen-3-one. Differences due to sex of bovine used were noted. These results confirm the ability of bovine liver to readily metabolize progesterone and form glucuronide conjugates of these compounds and suggest that adipose tissues take an active role in these actions in cattle.

INTRODUCTION

The use of progesterone in the animal industry today to control estrus (1) and initiate lactation (2) in cows, and to improve the rate of weight gain in steers (3) has led to the investigation of the eventual fate of this steroid in various tissues of the bovine, including the need to determine the functional role of these tissues in affecting hormone action.

The distribution of administered progesterone into various tissues of cattle has been reported (4) and the progesterone metabolites in muscle and adipose tissues (5) and in liver, kidney, and kidney fat tissues (6) of these same animals have been identified. These studies identified the metabolites of progesterone found in tissues following progesterone administration *in vivo*, but did not determine if these compounds were actually formed at that site or were carried to and deposited there by the blood. Lin *et al*. (5) observed a greater percentage of progestin conjugates in adipose tissues than in the plasma of these animals. This would suggest that adipose tissue is capable of forming water-soluble conjugates of steroids, since sequestering conjugates of steriods appears less likely.

In the present study, we have attempted to determine if bovine liver and adipose tissues indeed possess the intrinsic enzymatic capability to metabolize progesterone and to form glucuronide conjugates of these steroids, and also to identify the progesterone metabolites formed by each tissue.

MATERIALS AND METHODS

Three each of beef bulls, steers and cows (Angus, Hereford and Crossbred), and dairy cows (Holstein) were used in a completely randomized design with duplicate incubation samples used for each of the three types of tissues collected from each animal. Analysis of variance and Duncan's NMRT (7) were used to determine significant differences between groups concerning percentage conjugation and metabolites formed.

Tissue Preparation and Incubation Procedures

Tissue samples were obtained from cattle slaughtered at the Washington State University meats laboratory. Following bleeding, evisceration and hide removal, the liver, kidney fat, and subcutaneous fat tissue from the brisket area of each animal were collected. Tissues were then transported on ice to the laboratory for further preparation. Time from slaughter to beginning of incubation ranged from 2 to 3 hours.

Tissues were rinsed in ice cold physiological saline (0.9% NaCl) to remove any blood, and then finely minced with scissors. The minced tissues were carefully weighed into 10 g (liver) and 20 g (fat) samples and once again rinsed with ice cold physiological saline solution and placed on ice. Individual liver samples were homogenized in 66 ml ice cold Dulbecco's phosphate buffered saline, pH 7.4 (PBS) (Cat. #450-1300 GIBCO Laboratories, Grand Island, NY), and individual fat samples were homogenized in 40 ml PBS using a Sorval homogenizer. Differences in the amount of liver and fat homogenized was an attempt to decrease the difference in total protein of each tissue sample. Protein determinations were made using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA), using bovine gamma globulin as standard. The homogenates were centrifuged at 25,000 xg for 30 min in a refrigerated centrifuge. Supernatants containing a suspension of microsomes and cytosol were aspirated from beneath the fat layer and used for incubation. The fat layer and pellet were discarded.

Two milliliters of cold supernatant (or PBS for controls) were added to a 20 ml glass vial containing 2.69 µg [4-^{14}C]progesterone (0.11 µCi) substrate. Total supernatant protein added per vial was 61.5 mg, 12.0 mg, or 7.2 mg of liver, subcutaneous fat, or kidney fat preparation, respectively. At the beginning of the incubation period, 1 ml of PBS containing 1 mg UDP-glucuronic acid substrate and a NADPH generating system, was added to supernatant and PBS control samples which gave a final concentration of 0.5 mM NADP, 10.0 mM glucose-6-phosphate and 25.0 mM nicotinamide. The PBS control excluded endogenous enzymes and therefore demonstrated any metabolic artifacts imposed by the assay system. The incubation was carried out in a Dubnoff water bath shaker at 37°C in an air atmosphere for 5 hours. To terminate the incubation the samples were frozen at -20°C.

Extraction Procedures

Frozen incubation samples were allowed to thaw at room temperature after which ether extractable steroids were separated from unextractable steroids by extraction (3x) with two volumes of diethyl ether. The pooled extract was dried and redissolved in 95% ethanol and an aliquot was taken for counting in a Packard Tricarb 3330 liquid scintillation counter to determine the amount of radioactivity (free steroids) partitioned into the solvent phase. The scintillation cocktail used consisted of 4g Omnifluor (New England Nuclear, Boston, MA) and 10 ml ethanol per liter toluene. Ether unextractable radioactivity was considered as possible conjugated steroids left in the aqueous fraction. The aqueous fraction was frozen and saved for further analysis.

Hydrolysis of Conjugates

Frozen aqueous phase samples were thawed, pooled within animal groups, and again extracted (3x) with two volumes of diethyl ether to remove any steroids which possibly had been hydrolyzed during freezing, storage and thawing. The remaining aqueous phase was then submitted to steroid-glucuronide hydrolysis using β-glucuronidase (Type B-2: Bovine liver, Sigma Chemical Co., St. Louis, MO) as

previously described by Estergreen et al. (4). Hydrolyzed steroids were then extracted (3x) with diethyl ether and submitted to chromatography for identification of the steroids which had been conjugated as glucuronides.

Thin-layer Chromatography

Free and conjugated steroid samples in 95% ethanol were dried and applied with chloroform:methanol (1:1 v/v) to 10x20 cm glass plates coated with aluminum oxide (0.30 mm thick) for initial thin-layer chromatography (TLC). Appropriate progestin standards were applied to adjoining parallel lanes for comparison of chromatographic mobility with the sample steroids. The plates were placed in solvent tanks containing hexane:ethyl acetate (3:2 v/v) (solvent system A) and allowed to develop once. After plates were removed from the solvent tank they were allowed to dry and then were scanned with a radiochromatogram scanner to locate areas of radioactivity. Peak areas of radioactivity were removed by vacuum aspiration into glass pipet tubes containing a glass wool plug to trap the solid material. The radioactive steroid in each tube was then eluted with chloroform:methanol (1:1 v/v), dried and redissolved in 95% ethanol. An aliquot was taken and the amount of radioactivity determined by liquid scintillation counting for quantitative comparisons of each peak. The TLC plates were submitted to UV light to locate progestin standards containing a Δ^4-3-keto group, and then iodine vapor to locate other progestin standards. Mobility of each standard and sample peak was measured and related to progesterone mobility (Rp). Sample peak Rp's were then compared to standard Rp's for preliminary identification. Further identification of specific metabolites was accomplished using additional TLC with solvent systems of benzene:methanol (19:1 v/v) (solvent system B) and hexane:ethyl acetate (1:8 v/v) (solvent system C).

High Performance Liquid Chromatography

Following the initial TLC of free and conjugate diethyl ether extracts, those progestins of which enough material had been pooled and could be detected by UV absorption spectra were identified by high performance liquid chromatography (HPLC) (Spectra Physics 8000 HPLC, Santa Clara, CA) using a system similar to that reported by Purdy et al. (6).

Radioactive peaks, having been eluted from the initial TLC, were evaporated to dryness and redissolved in 20% isopropanol in heptane (2 ml). This was passed through a Sep-pak C_{18} cartridge (Waters Associates, Inc., Milford, MA) followed by an additional 3 ml solvent to remove any contaminating debris from the sample. The eluent was again evaporated to dryness and redissolved in the appropriate volume of 20% isopropanol in heptane for injection into a 0.46 x 30 cm Chromegabond Diol column (E. S. Industries, Marlton, NJ) equilibrated with 0.8% isopropanol in heptane. The column was then developed using a concave gradient (Non-linear gradient 2, Spectra Physics) of 0.8 to 7% isopropanol in heptane over a period of 1 hr at a flow rate of 2 ml/min. Fractions of 0.5 ml (15 sec) were collected and examined for

radioactivity. Retention times (RT) of the radioactive peaks were compared to RTs of non-radioactive standards detected by UV absorption at 254 nm.

Recrystallization to Constant Specific Activity

Further identification of the recovered progestins was accomplished by co-crystallization of an aliquot of pooled radioactive steroid with 10-25 mg of non-radioactive standard steroid to a constant specific activity. Solvent pairs were used for two consecutive crystallizations each in the order shown using the following combinations: benzene/hexane and acetone/pentane (progesterone and 20-hydroxy-4-pregnen-3-ones), chloroform/methanol and benzene/hexane (pregnanediones), chloroform/hexane and methylene chloride/petroleum ether (pregnanolones), and ethyl acetate/hexane and acetone/water (pregnanediols). The specific activity of consecutive crystallizations was not constant if the variation was greater than ±5% about the mean.

RESULTS

Conjugate Formation

Partitioning of steroids from incubated supernatant preparations of bovine liver tissue into ether extractable and unextractable (aqueous phase) fractions revealed that a substantial amount (44.7 ± 14.2%) (mean ± SD) of the original radioactivity was left in the aqueous phase presumably as conjugate (Table 1). Radioactivity also remained in the aqueous phase of subcutaneous fat preparations (5.0 ± 3.6%) and kidney fat preparations (3.7 ± 2.2%) following exhaustive extraction of free steroids with diethyl ether. Hydrolysis of the glucuronide conjugated steriods of both liver and adipose tissues with β-glucuronidase liberated most (78% in liver, 81% in adipose tissue) of this radioactivity when extracted with diethyl ether, suggesting a prior existence of these steroids as glucuronide conjugates. The use of the word "conjugate" hereafter refers to these steroids extracted following hydrolysis with β-glucuronidase, but does not imply absolute proof of prior conjugation.

Beef bull livers exhibited significantly greater ($P < 0.05$) conjugation of steroids than those of beef cows and beef steers, while dairy cow livers, exhibited significantly greater ($P < 0.05$) conjugate formation than those of beef steers (Table 1). Protein:steroid ratios of liver samples between animal groups were not statistically different ($P > 0.10$), and therefore do not account for the differences observed in conjugate formation. No significant differences ($P > 0.05$) in conjugate formation were observed in or among adipose tissues obtained from different animal groups. No conjugate formation was observed in controls.

Identification of Progesterone and its Metabolites

Positive identification of the free and conjugated progestins recovered from bovine liver and adipose tissues was accomplished by additional TLC or HPLC (Tables 2 and 3), and by recrystallization to

TABLE 1. The percentage ($\bar{x}$ ± S.D.) radioactivity left in aqueous phase of bovine tissue incubations following ether extraction.

Animals	Number of Animals	Liver	Subcutaneous fat	Kidney fat
Beef bulls	3	60.1 ± 14.0^{a}	7.8 ± 1.7^{a}	4.9 ± 2.4^{a}
Beef steers	3	28.7 ± 3.0^{c}	4.8 ± 3.5^{a}	2.5 ± 3.5^{a}
Beef cows	3	41.6 ± 7.5^{bc}	3.8 ± 2.1^{a}	3.5 ± 0.8^{a}
Dairy Cows	3	48.2 ± 8.7^{ab}	6.1 ± 6.9^{a}	2.8 ± 3.2^{a}
Overall Mean	12	44.7±14.2	5.0±3.6	3.7±2.2
Control	6	0.2 ±0.2[1]		

[1]In PBS, no tissue, all substrates and cofactors included.

[a,b,c]Means with common superscript in a column within each tissue are not statistically different ($P > 0.05$).

constant specific activity (Table 4). Thin-layer chromatography was used rather than HPLC for identifying compounds which could not be detected by UV absorption (pregnanediols) or of which only a small amount of material was available, since lesser amounts of steroid were lost on TLC than HPLC.

Liver. The initial TLC of the free and conjugated extracts from incubation samples containing liver homogenate supernatants revealed four peaks of radioactivity from the free fractions and three peaks from the conjugate fractions. Peak area I, the most polar material, contained the majority of the total radioactivity recovered in both the free and conjugate fractions (73% and 84%, respectively). The high levels of these polar metabolites, chromatographing similarly to a group of pregnanediol standards, demonstrate the capability of bovine liver tissue to readily metabolize progesterone in vitro. Additional chromatography to identify individual steroids from this material was done using TLC solvent system A (developed twice) and solvent system C, where detection of the pregnanediol standards could be accomplished by visualization in iodine vapor. Solvent system A divided the original peak area I of both the free and conjugate fractions into three separate metabolite peaks, the first of which was more polar than any of the pregnanediol standards and therefore remained unidentified. The second peak migrated only with 5β3α20α standard and was again clearly identified as this pregnanediol when rechromatographed in solvent system C. The third peak co-migrated with three pregnanediol standards in solvent system A (free sample Rp = 0.261, 5β3α20β Rp = 0.258, 5α3α20α Rp = 0.252, 5α3β20α Rp = 0.268; conjugate sample Rp = 0.230, 5β3α20β Rp = 0.230, 5α3α20α

TABLE 2. Identification of progesterone and its in vitro metabolites in "free" fractions of bovine tissue extracts by thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC).

Steroid Identified	Solvent System[a]	TLC Chromatogram No. 1 Extr.	TLC Chromatogram No. 1 Std.	TLC Chromatogram No. 2 Extr.	TLC Chromatogram No. 2 Std.	HPLC Extr.	HPLC Std.
		Rp[b]				RT[c]	
Liver							
5β DHP	A,B	1.22	1.22	1.02	1.03		
3β5β	A,B	0.74	0.73	0.85	0.84		
3α5β	A	0.43	0.42			27.5	27.4
5β3α20β	A,C	0.26	0.25	0.69	0.68		
5β3α20α	A,C	0.13	0.12	0.54	0.54		
Subcutaneous fat							
Progesterone	A	0.98[d]	1.00[e]			11.5	11.5
20α	A	0.45[d]	0.47[e]			34.5	34.5
20β	A	0.45[d]	0.47[e]			31.8	31.7
3α5β	A	0.45[d]	0.47[e]			28.8	28.8
Unidentified	A	0.45[d]	0.47[e]			25.3	24.5[f]
Kidney fat							
Progesterone	A	0.97[d]	1.00[e]			11.5	11.6
20α	A	0.47[d]	0.50[e]			33.3	33.5
20β	A	0.47[d]	0.50[e]			30.5	30.5
3α5β	A	0.47[d]	0.50[e]			27.8	27.6
Unidentified	A	0.47[d]	0.50[e]			24.3	23.6[f]

[a] TLC solvent system used: A = hexane:ethyl acetate, 3:2 (v/v); B = benzene:methanol 19:1 (v/v); c = hexane:ethyl acetate 1:8 (v/v).

[b] $\frac{\text{Sample distance from origin}}{\text{Progesterone distance from origin}}$

[c] Retention time in minutes.

[d] Individual peaks not clearly separated following first TLC.

[e] Rp value for 20β standard.

[f] RT value of 3β5α standard. Sample peak is unidentified also due to its inability to recrystallize with 3β5α standard to a constant specific activity.

TABLE 3. Identification of progesterone and its in vitro metabolites in "conjugate" fractions of bovine tissue extracts by thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC).

Steroid Identified	Solvent System[a]	TLC Chromatogram No. 1 Extr.	TLC Chromatogram No. 1 Std.	TLC Chromatogram No. 2 Extr.	TLC Chromatogram No. 2 Std.	HPLC Extr.	HPLC Std.
		Rp[b]				RT[c]	
Liver							
Progesterone	A,B	1.01	1.00	0.97	1.00		
3α5β	A	0.55	0.57			28.8	28.8
5β3α20β	A,C	0.23	0.23	0.69	0.70		
5β3α20α	A,C	0.13	0.12	0.48	0.47		
Subcutaneous fat							
Progesterone	A,B	1.00	1.00	0.99	1.00		
Kidney fat							
Progesterone	A,B	1.01	1.00	1.01	1.00		
Subcutaneous fat + Kidney fat							
20α	A	0.53[d]	0.60[e]			33.0	33.0
20β	A	0.53[d]	0.60[e]			29.8	29.8
3α5β	A	0.53[d]	0.60[e]			27.3	27.1[f]
Unidentified	A	0.53[d]	0.60[e]			25.3	24.5[f]

[a] TLC solvent system used: A = hexane:ethyl acetate, 3:2 (v/v); B = benzene:methanol 19:1 (v/v); C = hexane:ethyl acetate 1:8 (v/v).

[b] $\frac{\text{Sample distance from origin}}{\text{Progesterone distance from origin}}$

[c] Retention time in minutes.

[d] Individual peaks not clearly separated following first TLC.

[e] Rp value for 20β standard.

[f] RT value of 3β5α standard. Sample peak is unidentified also due to its inability to recrystallize with 3β5α standard to a constant specific activity.

TABLE 4. Recyrstallization of recovered progestins from "free" and "conjugate" fractions of bovine tissue extracts to a constant specific activity.

Steroid	Fraction	Crystallization No. 1	2	3	4
		cpm/mg			
Liver					
5β DHP	Free	2420	2400	2420	2390
3β5β	Free	671	638	651	660
3α5β	Free	4860	4830	4810	4840
5β3α20β	Free	4640	4540	4760	4560
5β3α20α	Free	5230	4970	5290	5190
Progesterone	Conjugate	689	679	706	692
3α5β	Conjugate	4120	4180	4060	4120
5β3α20β	Conjugate	4020	3920	3970	3970
5β3α20α	Conjugate	5610	5690	5640	5520
Subcutaneous fat					
Progesterone	Free	5410	5380	5380	5330
20α	Free	1510	1490	1520	1520
20β	Free	2920	2950	2920	2940
3α5β	Free	2740	2740	2680	2760
Progesterone	Conjugate	749	737	751	751
Kidney fat					
Progesterone	Free	5570	5510	5490	5540
20α	Free	1940	1910	1970	1860
20β	Free	2970	3070	3020	3070
3α5β+	Free	1720	1740	1740	1730
3β5α+	Free	767	581	256	143
Progesterone	Conjugate	887	866	872	887

+Variation greater than ±5% around the mean.

Rp = 0.222, 5α3β20α Rp = 0.242), but was clearly identified as 5β3α20β in solvent system C (free sample Rp = 0.686, 5β3α20β Rp = 0.682, 5α3α20α Rp = 0.673, 5α3β20α Rp = 0.595; conjugate sample Rp = 0.692, 5β3α20β Rp = 0.699, 5α3α20α Rp = 0.675, 5α3β20α Rp = 0.641) (Tables 2 and 3).

Initial TLC peak area II, of both the free and conjugate fractions, demonstrated chromatographic mobility similar to 3α5β.

Additional identification of this peak as 3α5β was provided by HPLC (Tables 2 and 3). Peak area III of the initial TLC chromatogram from free and conjugate fractions, was submitted to a second TLC using solvent system B and not HPLC due to the small amount of material represented by this peak. These chromatographic results identified this peak as 3β5β in the free fraction, and as progesterone in the conjugate fraction (Tables 2 and 3). Peak area IV of the free fraction was identified as 5β DHP following TLC in both solvent systems A and B. This metabolite also comprised a low percentage of the total free radioactivity and therefore was not applied to HPLC. The radioactive residue left from each progestin identified by TLC or HPLC was then recrystallized to a constant specific activity with appropriate nonradioactive standard compounds for positive identification of progesterone and its metabolites formed in vitro (Table 4).

In summary, the metabolites of progesterone recovered following chromatography and identified in the free fraction of liver tissue preparations include 5β3α20α (46% of the recovered free radioactivity of liver), 5β3α20β (15%), 3α5β (22%), 5β DHP (4%), 3β5β (1%), and an unidentified metabolite (12%) which was more polar than any of the pregnanediols as determined by TLC. The conjugate progestins of liver included 5β3α20α (55% of the recovered conjugate radioactivity of liver), 5β3α20β (15%), 3α5β (13%), progesterone (2%), and an unidentifed metabolite (15%) which was also more polar than any of the pregnanediols as determined by TLC.

Adipose Tissues. Following incubation of the supernatants from subcutaneous and kidney fat samples, the initial TLC of extracts

demonstrated substantial metabolism of progesterone to a group of monohydroxy metabolites in both the free (13% and 9% of the recovered free radioactivity from subcutaneous fat and kidney fat, respectively) and conjugated fractions (12% and 9% of the recovered conjugate radioactivity). Progesterone found in the free fractions was confirmed by HPLC (Figure 1), whereas conjugated progesterone was further identified using TLC solvent system B (Tables 2 and 3).

The free monohydroxy metabolites from subcutaneous and kidney fat, and the combined conjugated monohydroxy metabolites of both tissues, were applied to HPLC for identification. Figure 1 illustrates the adipose tissue progesterone and monohydroxy peak composite profile when applied to the Chromegabond Diol column for HPLC. This system offered greater resolution of the monohydroxy metabolites than could be obtained by TLC. The metabolites in the free and conjugate fractions were tentatively identified as 3α5β, 20β and 20α. A fourth peak, running closest to 3β5α than other progesterone metabolites yet with a consistently longer retention time than the standard compound, remains unidentified due to its inability to recrystallize to a constant specific activity with non-radioactive 3β5α standard. Further chromatography of this peak revealed it as unlike any of the possible pregnanolone or pregnanediol metabolites of progesterone. The progestins which were identified by TLC or HPLC, were then recrystallized to constant specific activity with appropriate non-radioactive standards for confirmation of their identity except for the conjugate monohydroxy metabolites due to the loss of this radioactivity from HPLC (Table 4). Because of similarities in HPLC identification of these conjugate metabolites with those of the free fractions, they may indeed be the same.

In summary, the progestins recovered following chromatography from the free and combined conjugated fractions of subcutaneous and kidney fat in vitro preparations were identified as progesterone (87%, 91%, and 84% of the subcutaneous fat free fractions, kidney fat free fractions and combined conjugate fractions, respectively) 3α5β (5%, 1%, and 5%), 20β (4%, 4%, and 6%), 20α (1%, 1%, and 1%), and a metabolite which chromatographed close to 3β5α (3%, 3%, and 5%) following HPLC. The latter metabolite was not 3β5α as shown by its inability to co-crystallize to a constant specific activity with standard 3β5α (Table 4).

PBS Control. Only unchanged progesterone was found in ether extracts of non-tissue PBS controls, indicating no metabolism of progesterone occured due to incubation conditions or constitutents other than tissue (Table 5).

Differences in Progesterone Metabolism Among Animal Groups

Quantitative comparisons between beef bulls, beef steers, beef cows, and dairy cows were made concerning the extent of progesterone reduction which had occurred, as determined by the steroids recovered from the free fraction following initial TLC. A significant difference ($P < 0.05$) was noted between animal group liver samples. Dairy cow liver preparations exhibited the greatest amount of progesterone reduction to the fully saturated pregnanediol peak area I of the initial TLC chromatogram, while beef bull and beef steer livers formed the least amount of this highly polar material (Table 5). Protein:steroid ratios of liver samples between animal groups were not statistically different ($P > 0.10$), and therefore do not account for

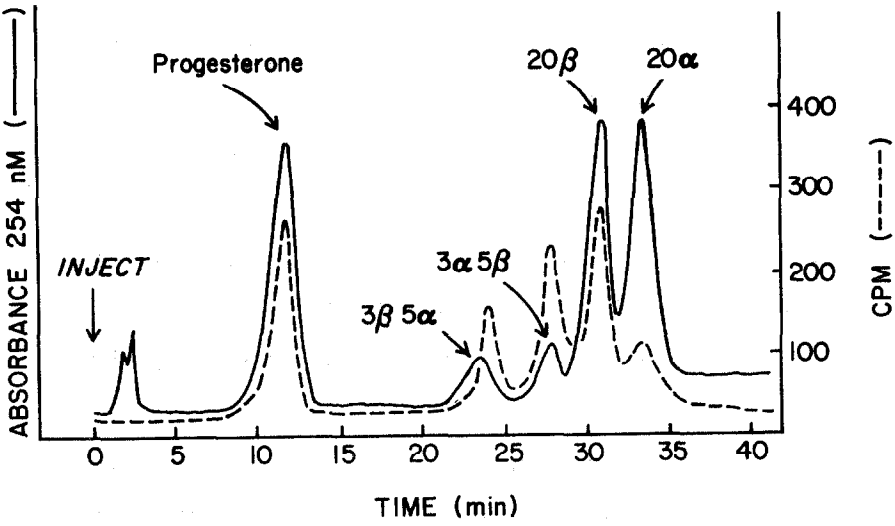

FIGURE 1. Composite diagram demonstrating HPLC separation and identification of [4-^{14}C]progesterone and its monohydroxy metabolites from bovine adipose tissue extracts. Metabolites were separated on a 0.46 x 30 cm Chromegabond Diol column developed using a concave gradient solvent system of 0.8% to 7.0% isopropanol in heptane over a period of 1 hr at a flow rate of 2 ml/min.

the differences observed in progesterone metabolism. No significant differences in the amount of progesterone reduced by adipose tissues were observed among animal groups.

DISCUSSION

Although the metabolism of progesterone in reproductive tissues of cattle appears to be fairly well documented, little has been reported on the ability of non-reproductive tissues to metabolize this steroid. The present report demonstrates for the first time that both liver and adipose tissues of cattle indeed possess the intrinsic

TABLE 5. The percentage ($\bar{x}$ ± S.D.) "free" progesterone and its most fully reduced *in vitro* metabolites recovered from bovine tissue preparations following initial TLC, demonstrating differences in the extent of progesterone metabolism among animal groups.

Tissue	TLC Fractions[1]	Controls[2] (6)	Beef Bulls (3)	Beef Steers (3)	Beef Cows (3)	Dairy Cows (3)	Overall Mean (12)
Liver	I	100.0	0.0	0.0	0.0	0.0	0.0
	II	0.0	28.6±3.6^{a}	25.1±3.8^{ab}	20.3±4.7^{ab}	15.0±7.9^{b}	22.2±7.1
	III	0.0	66.9±5.6^{b}	68.3±2.9^{b}	76.1±6.7^{ab}	79.9±7.4^{a}	72.8±7.6
Subcutaneous fat	I	100.0	81.2±5.3^{a}	88.7±2.7^{a}	95.9±1.6^{a}	80.3±18.7^{a}	86.5±10.7
	IV	0.0	18.9±5.3^{a}	11.6±2.2^{a}	4.1±1.6^{a}	17.9±15.8^{a}	13.1±9.5
Kidney fat	I	100.0	83.0±9.4^{a}	94.8±4.5^{a}	97.9±1.9^{a}	87.3±13.3^{a}	90.7±9.5
	IV	0.0	16.1±7.8^{a}	5.2±4.5^{a}	2.1±1.9^{a}	11.5±5.4^{a}	8.7±8.4

1. I. Behaved chromatographically like progesterone.
   II. Behaved chromatographically like 3α5β.
   III. Behaved chromatographically like pregnanediols.
   IV. Behaved chromatographically like monohydroxy metabolites.

2. In PBS, no tissue, all substrates and cofactors included.

^{ab}Means with common superscript within a row are not statistically different ($p > 0.05$).

enzymatic capability to metabolize progesterone in vitro. The most dramatic progesterone metabolizing activity was observed in the bovine liver, as evidenced by the high level (67-80% of total radioactivity) of the fully saturated progesterone metabolites, the pregnanediols, which appeared following incubation. Subcutaneous and kidney fat metabolized a substantial amount (2-19%) of progesterone to a group of monohydroxy metabolites.

Progesterone metabolites in the bovine liver appear exclusively as 5β pregnanes (Tables 2 and 3) (6). Among the metabolites identified from bovine liver incubations were 3α5β and 5β3α20β, both of which have previously been isolated in vivo from bovine livers by Purdy et al. (6), the latter metabolite also appearing in bile of pregnant cows (8). Neither of these studies reported finding 5β3α20α, a pregnanediol which was the major metabolite formed by bovine liver preparations in the present study, and is a major excretory product in cattle (9). The other metabolites identified in vitro, which are most likely intermediates to the pregnanediols identified, include 5β DHP and 3β5β. It would appear from the absence of 20α and 20β that progesterone metabolism in bovine liver is initiated by enzymes catalyzing the reduction of ring A, followed by reduction of the 20 ketone as has been previously reported in livers of the rat (10).

Lin et al.(5) have identified the metabolites of progesterone in the subcutaneous fat of cattle following progesterone administration. However, they did not determine if the metabolites were actually formed by enzyme systems inherent to the tissue or if they were formed elsewhere and were sequestered in the adipose tissues from the blood system. We have now demonstrated that bovine adipose tissues indeed

possess intrinsic enzyme systems for synthesizing the progesterone monohydroxy metabolites 3α5β, 20β and 20α. These metabolites were also isolated *in vivo* by Lin *et al.* (5) along with 3α5α, 3β5β and 20α5α which were not observed *in vitro*.

Although extensive progesterone metabolism by the liver has been reported in other animal species (10,11), few investigators have observed adipose tissues actively metabolizing steroids. Wiest (12) reported the metabolism of progesterone to 20α by peritoneal fat of the rat. More recently, Frost *et al.* (13) demonstrated that human adipose tissue can convert androstenedione to estrone, *in vitro*. These reports, along with the present study, provide further evidence that adipose tissues can metabolize steroids. This activity may be of major importance in progesterone metabolism due to the large quantity of adipose tissue in most animals and its ability to sequester large amounts of progesterone from the blood (14).

Apparent progestin-glucuronide conjugates were observed in bovine liver (29-60%) and adipose tissue (3-8%) preparations following 5 hr of incubation. Substantial conjugate formation may be expected in the livers of these animals, however, the capability of adipose tissues to actively form water-soluble progestin-conjugates had not been demonstrated previously. Glucuronyltransferase activity has, however, been located in retroperitoneal fat of the rat using 4-methylumbelliferone as substrate (15). Lin *et al.* (5) reported finding about one-third of the recovered progestins in subcutaneous fat of progesterone-treated cattle as water-soluble conjugates, suggesting that these tissues may possibly contain enzyme systems capable of catalyzing steroid conjugating activity. Our results now give strong evidence for the presence of such enzyme systems in bovine adipose tissue.

The majority of the conjugated steroids released by β-glucuronidase hydrolysis from adipose tissue preparations was progesterone. A small amount of an apparent progesterone-glucuronide was also observed in the liver incubation samples. Although some free progesterone may have remained bound to protein in the aqueous phase following exhaustive ether extraction, it appears that the additional steroids extracted after hydrolysis were released by the β-glucuronidase. These observations suggest, but do not prove, an unusual capability of bovine adipose and liver tissues to conjugate glucuronic acid to a ketone of progesterone, a reaction that has usually been considered unlikely under normal physiological conditions. Apparent progesterone-glucuronides were also identified by Lin et al. (5) in bovine adipose tissue in vivo. Steroid-enol-glucuronides have been reported by Wotiz and Fishman (16) in rat urine.

Significant differences were observed between animal groups in both progesterone metabolism and the conjugation of these steroids by liver. Although the actual factors influencing these differences were not determined, the possibility of them being sex-linked should be taken into consideration since differences due to sex have been reported in rat livers for both progesterone metabolism (17, 18) and progestin-glucuronide conjugation enzyme activity (19).

Thus, it appears that adipose tissues, as well as liver tissues of cattle, may be involved in the structural alteration of progesterone via metabolism and/or conjugation, and that differences in these actions may occur in the liver considering the sex and type of bovine studied.

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REFERENCES

1. Trimberger, G. W., and Hansel, W.; J Anim Sci 14: 224 (1955).
2. Smith, K.L., Muir, L. A., Ferguson, L. C., and Conrad, H. R.; J Dairy Sci 54: 1886 (1971).
3. Deans, R.I., Van Arsdell, W. J., Reineke, E. P., and Bratzler, L. J.; J Anim Sci 15: 1020 (1956).
4. Estergreen, V. L., Lin, M. T., Martin, E. L., Moss, G. E., Branen, A. L., Luedecke, L. O., and Shimoda, W.; J Anim Sci 45: 642 (1977).
5. Lin, M. T., Estergreen, V. L., Moss, G. E., Willett, J. D., and Shimoda, W.; Steroids 32: 547 (1978).
6. Purdy, R. H., Durocher, C. K., Moore, P. H., Jr., and Rao, P. N.; J Steroid Biochem 13: 1307 (1980).
7. Steel, R. G. D., and Torrie, J. H., Principles and Procedures of Statistics, ed. 2. McGraw-Hill Book Co., New York p 187 (1980).
8. Pearlman, W. H., and Cerceo E.; J Biol Chem 176: 847 (1948).
9. Wright, A. A.; Vet Rec 70: 662 (1958).
10. Crane, M., Loring, J., and Villee, C. A., Endocrinology 87: 80 (1970).
11. Chatterton, R. T., Jr,and Chatterton, A. J., and Hellman, L; Endocrinology 87: 941 (1970).
12. Wiest, W. G.; Endocrinology 73: 310 (1963).
13. Frost, P. G., Reed, M. J., and James, V. H. T.; J Steroid Biochem 13: 1427 (1980).
14. McCracken, J. A.; J Endocrinol 28: 339 (1964).
15. Aitio, A; Int J Biochem 5: 325 (1974).
16. Wotiz, H. H., and Fishman, W. H.; Steroids 1: 211 (1963).
17. Yates, F. E., Herbst, A. L., and Urquhart, J.; Endocrinology 63: 887 (1958).
18. Einarsson, K., Gustafsson, J-A., and Goldman, A.S.; Eur J Biochem 31: 345 (1972).
19. Rao, L. G. S., and Taylor, W.; Biochem J 96: 172 (1965).

Abbreviations used: 5β DHP, 5β-pregnane-3,20-dione; 3α5α, 3α-hydroxy-5α-pregnan-20-one; 3α5β, 3α-hydroxy-5β-pregnan-20-one; 3β5α, 3β-hydroxy-5α-pregnan-20-one; 3β5β,3β-hydroxy-5β-pregnan-20-one; 20α, 20α-hydroxy-4-pregnen-3-one; 20β,20β-hydroxy-4-pregnen-3-one; 20α5α, 20α-hydroxy-5α-pregnan-3-one; 5β3α20α, 5β-pregnane-3α,20α-diol; 5β3α20β, 5β-pregnane-3α,20β-diol; and 5α3β20α, 5α-pregnane-3β,20α-diol; and 5α3α20α, 5α-pregnane-3α,20α-diol.