Metabolism of Progesterone in the Canine Feto-Placental Unit (43192)

RALPH A. KINSELLA, JR.,*[,1] GERHARD H. MUELHEIMS,* AND FAITH ELLEN FRANCIS[†]
Department of Internal Medicine, Department of Obstetrics and Gynecology,[†] and Edward A. Doisy Department of Biochemistry,[†] St. Louis University School of Medicine, St. Louis, Missouri 63104*

Abstract. **Progesterone (P), a major hormone of pregnancy, was selected for study under the conditions of an intact utero-placental-fetal unit. A preparation of the canine gravid uterus, near term, is described and shown to permit observation of the metabolic relationships of the steroid hormone P between maternal and fetal organisms. [1,2-^{3}H] P or [7α-^{3}H]P was injected into pups, while [4-^{14}C]P was injected into the uterine circulation. Perfusion was continued for 1 hr with excellent survival of the pups. Identified metabolites in the fetal and maternal tissues suggest a metabolic pool that is shared throughout the unit.** [P.S.E.B.M. 1991, Vol 196]

The metabolism of progesterone (P), a major hormone of pregnancy, has been studied in the isolated gravid canine uterus. The gravid uterus is the minimal organization containing fetal, placental, and maternal metabolic units. As an isolated preparation, it lends itself to investigations in which both the fetal and maternal circulatory relationships of the placenta are preserved. An important respect in which it differs from *in vitro* studies of tissues and perfusion of the fetus and placenta after their removal from the uterus (1, 2) is that it permits observation of movement of compounds from the maternal circulation to the placenta and fetus and vice versa. Early studies (3) had established that the gravid canine uterus may be maintained for several hours in terms of survival of the pups by means of perfusion of the uterus utilizing a pump-oxygenator.

Materials and Methods

Procedure for Perfusion. Four healthy pregnant mongrel bitches were used, each of which weighed approximately 25 kg and was 5–7 days from term. Under intraspinal lidocaine anesthesia, the uterus was delivered through a mid-line incision, enveloped in a plastic bag, and kept warm with warm towels. The uterine circulation was isolated as follows. A segment of the aorta was isolated so as to preserve ovarian and uterine arteries, while all other vessels were ligated. The right external iliac artery was taken for perfusion and the left external iliac artery was used for recording arterial pressure. The inferior vena cava was isolated as for the aorta except that the left renal vein was preserved for the effluent perfusate.

The perfusate was heparinized male canine whole blood oxygenated by means of a disposable Perfuso-Pac (Travenol Laboratories, Inc., Morton Grove, IL). Pumping was carried out with a calibrated Sigmamotor model TM 10 pump. The pumping rate, adjusted to maintain pressure at the level found at the beginning of the surgical preparation, 125 mm Hg, was 125–130 ml/min.

Injection of [^{3}H]P and [^{14}C]P. Four experiments were performed. In three experiments, [^{3}H]P was injected into the pups, while [^{14}C]P was introduced into the perfusate. In a fourth experiment, [^{14}C]P was added to the perfusate only.

In Experiments 1 and 2, 250 μCi of [1,2-^{3}H]P (sp act, 34 Ci/mmol) and in Experiment 3, 250 μCi of [7α-^{3}H]P (sp act, 20 Ci/mmol) (New England Nuclear Research Products, Du Pont Co., Wilmington, DE) were dissolved in 2 ml of 95% ethanol. Ten minutes after beginning pump perfusion of the uterus, 1 ml was injected into each of two pups at the ovarian ends of the uterine horns. The injections were made intracoelomically in Experiment 1 and intrathoracically in Experiments 2 and 3. In all four experiments, 10 μCi of [4-^{14}C]P (sp act, 57 mCi/mmol), dissolved in 1 ml of propylene glycol:95% ethanol (9:1), was injected into

[1] To whom requests for reprints should be addressed at St. Louis University Hospital, 3635 Vista Avenue at Grand Boulevard, P.O. Box 15250, St. Louis, MO 63110-0250.

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the tubing near its connection with the right iliac artery. The timing of this injection averaged 17 min from initiation of the pump perfusion.

After continuing the perfusion for 60 min, the uterus was opened, the pups were delivered, and the perfusion was stopped. In the three experiments utilizing [^{3}H]P and [^{14}C]P, all of the pups were alive. The number of pups delivered in Experiments 1, 2, and 3 were 5, 10, and 7, respectively. In the experiment in which only [^{14}C]P was added to the perfusate, six of the eight pups delivered were alive.

Collection, Extraction, and Fractionation of Tissues. The pups were killed by decapitation, dissected, and their organs and placentas were pooled according to origin from injected or uninjected pups. For example, a pool was made of livers from injected pups and a second similar pool was made of livers from uninjected pups. The pools were subjected immediately to sequential extraction using methylal:methanol (4:1) (4), then ethyl acetate. Maternal uterine tissue was extracted in the same way. The perfusate remaining after termination of the experiment was centrifuged and the plasma obtained was extracted sequentially with dichloromethane, then ethyl acetate.

Essentially, the methods employed for purification, fractionation, gas chromatography (GC), and high-performance liquid chromatography (HPLC) were those described previously (5, 6). The following modifications were introduced.

Residues of extracts of organs were chromatographed on columns of florisil:celite (3:1) (florisil 200–300 mesh; FLR-Floridin Co., Berkeley Springs, WV; celite 545; Johns-Manville, Wakegan, IL) and eluted with successive increases in percentage of ethyl acetate in benzene. The elutriates containing radioactivity were pooled and the residues of these elutriates were subjected to a mild Girard's T reagent procedure (7). This reaction was carried out at 37°C for 18 hr. For further fractionation of elutriates containing radioactivity, ascending thin-layer chromatography (TLC) was employed. The TLC was performed on silica impregnated glass fiber sheets (Chrom AR 1000; Mallinckrodt, St. Louis, MO) 36 cm in length in the solvent systems, 17% ethyl acetate in benzene for ketonic material, and 5% ethanol in benzene for nonketonic material. As indicated in Table I, some nonketonic fractions were chromatographed on columns of alumina before TLC was carried out. Thin-layer chromatography of elutriates containing radioactivity was then performed.

The method for GC was modified to include a radioactive monitor attachment and the combustion system described by Swell (8). The GC phase and temperatures were 3% QF-1 (Applied Science, State College, PA) at 225°C or 240°C. Quantitation was carried out by means of planimetry from GC tracings and was expressed as percent of the total radioactivity present in the appropriate segment cut from the thin-layer chromatogram.

Fractions of 1 ml were collected from the HPLC column with a FRAC-100 (Pharmacia, Inc., Piscataway, NJ) fraction collector. The elutriates were evaporated under a stream of nitrogen in a warm water bath, and aliquots of the residues were assayed for radioactivity.

For further identification of pregnanediols and pregnanolones, residues of elutriates of separate tissues were subjected to HPLC, and the appropriate fractions were collected and pooled with respect to each steroid. Pools of each pregnanediol and pregnanolone were then oxidized with CrO_3 (9), and the products were analyzed by HPLC.

Radioactivity was measured in a Mark 111 6880 series Liquid Scintillation Counting System (TM Analytic, Inc., Elk Grove Village, IL) using a solution of toluene containing 0.3% 2,5-diphenyloxazole and 0.03% 1,4-bis-[3-(5-phenyloxazolyl)]-benzene at efficiencies of 56% for ^{3}H and 95% for ^{14}C.

The ^{3}H- and ^{14}C-labeled steroids used in the experiment were purified by TLC in the system 3% ethanol in benzene and by paper chromatography in the system petroleum ether (boiling point, 64–67°C)-propylene glycol (10). The reference steroids used for chromatography were obtained from commercial sources. Their purity was evaluated as described previously (5) and by HPLC.

Results

The compounds identified in various organs did not vary significantly among the four experiments. In the experiment in which only [^{14}C]P was introduced into the perfusate, the distribution of the isotope among identified compounds in uninjected liver and placenta, maternal uterus, and terminal perfusate was the same as its distribution in these organs in the three experiments in which [^{3}H]P was also injected into the pups. Therefore, the data presented in Table I are a pool of the data obtained for all of the experiments. The data in Table II are a pool of the results obtained in the three experiments in which both [^{3}H]P and [^{14}C]P were utilized.

Identification of Metabolites. Metabolites of P and the organs in which they were present are listed in Table I. Criteria for identification of the metabolites and prepared derivatives were retention times in several chromatographic systems compared with reference compounds. The systems were GC, and HPLC in two systems for the metabolites as described previously (6), and the derivatives were CrO_3 oxidation products of the pregnanediols and of the principal pregnanolones identified with the HPLC solvent systems.

Initially, the distribution of isotopic labels was determined in most fetal organs. In general, those chosen

Table I. Metabolites of Progesterone

Organ[c]	Compounds[a], criteria of identification[b]																							
	P			Pregnanolones											5α-P′dione			5β-P′dione			5β,3α,20β-P′diol			5α,3β,20β-P′diol		
				5β,3α			5α,3β			5α,3α			5β,3β													
	HPLC	GC	other	HPLC	GC	other	HPLC	GC	other	HPLC	GC	other	HPLC	GC	other	HPLC	GC	other	HPLC	GC	other	HPLC	GC	other	HPLC	GC	other
Injected liver	+	+		+	+	CrO_3	+	+	CrO_3	+	+		−	−		−			−			+	+	CrO_3 Al	+	+	CrO_3 Al
Uninjected liver		−			+			+			+			−			−			−			+	+		Al	+	+	CrO_3 Al
Injected intestine	+	+		+	+	CrO_3	+	+	CrO_3	+	+		+	+		+	+		−	−							
Injected placenta	+	+		+	+	CrO_3	+	+	CrO_3	+	+		+			+			−			+	+	CrO_3 Al	+	+	CrO_3 Al
Uninjected placenta		+			+			+			+			−			+			−			+	+	CrO_3 Al	+	+	CrO_3 Al
Injected lung	+	+		+	+	CrO_3	+	+	CrO_3	+	+		+			+	+		−	−							
Injected brain	+	+		+	+		+	+	CrO_3	+	+		+	+		+	+		−	−							
Maternal uterus	+	+		+	+	CrO_3	+	+	CrO_3	+	+		+			+	+		+	+		+	+	CrO_3 Al	+	+	CrO_3 Al
Terminal perfusate	+	+		+	+	CrO_3	+	+			+		+	+		+	+		+	+		+	+		Al	+	+	Al

[a] 5β,3α = 3α-OH-5β-pregnan-20-one; 5α,3β = 3β-OH-5α-pregnan-20-one; 5α,3α = 3α-OH-5α-pregnan-20-one; 5β,3β = 3β-OH-5β-pregnan-20-one; 5α-P′dione = 5α-pregnane-3,20-dione; 5β-P′dione = 5β-pregnane-3,20-dione; 5β,3α,20β-P′diol = 5β-pregnane-3α,20β-diol; 5α,3β,20β-P′diol = 5α-pregnane-3β,20β-diol.

[b] Criteria of identification refers to identity with the reference steroid carried through the procedure, as described in the text. CrO_3 = identity with the expected product following oxidation with CrO_3 of appropriate fractions separated by HPLC; Al = presence of compound in the 3% ethanol in benzene elutriate following chromatography on columns of alumina; + = identity with the reference steroid; − = not detected.

[c] Injected organs = organs and attached placentas recovered from the two pups at the ovarian ends of the uterine horns that were injected with [^{3}H]progesterone; uninjected organs = organs and attached placentas recovered from the remaining pups.

Table II. Distribution of Metabolites of Progesterone

Organ	Compound																	
	P		Pregnanolones								5α-P′dione		5β-P′dione		5β,3α,20β-P′diol		5α,3β,20β-P′diol	
			5β,3α[a]		5α,3β		5α,3α		5β,3β									
	% of ^3H[b]	% of ^{14}C	% of ^3H	% of ^{14}C	% of ^3H	% of ^{14}C	% of ^3H	% of ^{14}C	% of ^3H	% of ^{14}C	% of ^3H	% of ^{14}C	% of ^3H	% of ^{14}C	% of ^3H	% of ^{14}C	% of ^3H	% of ^{14}C
Injected liver	2.8	8.1	9.2	23.4	3.1	8.2	1.2	2.7	—	—	—	—	—	—	53.3	38.3	30.5	19.3
Uninjected liver	—	—	7.1	6.7	2.4	4.1	2.0	1.2	—	—	—	—	—	—	56.2	58.7	32.3	29.3
Injected intestine	Tr[c]	*[d]	16.4	*	58.1	*	12.7	•	7.2	*	5.7	*	—	*				
Injected placenta	8.1	10.9	28.4	21.2	34.0	32.9	5.5	8.2	Tr	Tr	Tr	Tr	—	—	12.4	12.5	11.6	14.3
Uninjected placenta	9.3	12.9	16.6	15.3	32.4	39.2	7.0	10.1	—	—	1.0	3.6	—	—	19.7	11.5	14.0	7.5
Injected lung	Tr	•	46.5	*	38.8	•	14.7	•	Tr	*	Tr	*	—	*				
Injected brain	Tr	*	14.5	*	56.1	•	4.6	*	5.6	•	19.1	*	—	*				
Maternal uterus	Tr	Tr	19.4	8.0	38.2	23.8	9.4	36.5	Tr	Tr	7.7	10.7	7.7	11.9	9.5	4.8	8.0	4.2
Terminal perfusate	16.8	38.0	28.3	18.3	8.4	12.5	Tr	Tr	Tr	Tr	9.4	8.1	7.4	6.4	20.4	12.7	9.2	4.1

[a] Abbreviations for compounds are defined in the footnotes of Table I.
[b] % of ^3H = percentage of the total identified radioactivity in each organ labeled with ^3H contributed by the detected steroid; % of ^{14}C = percentage of the total identified radioactivity in each organ labeled with ^{14}C contributed by the detected steroid. For the organs injected intestine, injected lung, and injected brain, the total identified radioactivity labeled with ^3H or with ^{14}C is that contributed by the ketonic fractions only. The nonketonic fractions were not analyzed.
[c] Tr = presence of less than 1% of the detected steroid.
[d] * = insufficient ^{14}C-labeled disintegrations per minute for quantitation by GC.

subsequently for detailed study are tissues commonly implicated in steroid hormonal metabolism which contained sufficient amounts of radioactivity to permit fractionation and analysis. Nonketonic fractions of intestine, lung, and brain from injected pups were not analyzed for individual compounds. For the identification of P and metabolites, 3α-hydroxy-5β-pregnan-20-one, 3β-hydroxy-5α-pregnan-20-one, 3α-hydroxy-5α-pregnan-20-one, 5β-pregnane-3α,20β-diol, and 5α-pregnane-3β,20β-diol, the primary criterion was GC. High-performance liquid chromatography was employed in most instances to confirm the results of GC. In a few instances, TLC fractions expected to contain 3β-hydroxy-5β-pregnan-20-one, 5α-pregnane-3,20-dione, and 5β-pregnane-3,20-dione were analyzed only by HPLC.

Oxidation with CrO_3 of pools of 3α-hydroxy-5β-pregnan-20-one or of 5β-pregnane-3α,20β-diol yielded the single expected product, 5β-pregnane-3,20-dione, identified by HPLC. Similarly, oxidation of pools of 3β-hydroxy-5α-pregnan-20-one or of 5α-pregnane-3β,20β-diol yielded the single product, 5α-pregnane-3,20-dione.

Following chromatography of nonketonic fractions on columns of alumina, 5β-pregnane-3α,20β-diol and 5α-pregnane-3β,20β-diol were identified by GC and HPLC in the 3% ethanol in benzene elutriates.

Other steroids not listed in Table I were identified in very small amounts in various organs. Using HPLC as the criterion, traces of 5β-pregnane-3α,20α-diol and 5α-pregnane-3β,20α-diol were identified only in the terminal perfusate. 20α-Hydroxy-5β-pregnan-3-one was identified in injected placenta, and 20α-hydroxy-5α-pregnan-3-one was identified in injected intestine and maternal uterus. The isomers 20β-hydroxy-5β-pregnan-3-one and 20β-hydroxy-5α-pregnan-3-one were not detected in any organ.

In general, analysis of elutriates of TLC sheets expected to contain compounds more polar than 3α-hydroxy-5β-pregnan-20-one and pregnanediols was not undertaken. However, using GC and HPLC as criteria, a search was carried out for 20α-hydroxy-4-pregnen-3-one and 20β-hydroxy-4-pregnen-3-one in placentas, livers, and terminal perfusate. Traces of both steroids were identified in injected and uninjected placentas and in injected liver. Traces of 5β-pregnane-3α,17α,20α-triol were identified in injected and uninjected placentas and livers, as well as in the terminal perfusate. Unindentified pregnanetriols were also detected in these organs.

Distribution of Radioactivity among Metabolites. Table II presents the percentage of the total identified radioactivity labeled with ^3H and the percentage of the total identified radioactivity labeled with ^{14}C contributed by the detected steroid. Unidentified radioactivity represented the elutriates of TLC sheets not analyzed, principally containing material more polar than pregnanolones and pregnanediols. A small amount of radioactivity was present also in elutriates containing material less polar than pregnanediones and pregnanediols. In intestine, lung, and brain from the injected pups, the very low levels of ^{14}C represented by individual compounds precluded quantitative determination by GC.

Identified radioactivity in the organs of the pups and in the maternal uterus did not include large amounts of unmetabolized [^3H]P or [^{14}C]P. The amount of unmetabolized P ranged from 9.3% of the ^3H- and 12.9% of the ^{14}C-identified radioactivity in uninjected placenta to undetectable amounts in uninjected liver. Larger amounts of [^3H]P and, particularly, [^{14}C]P were found in the terminal perfusate. It is of interest that tissue from sites of injection of [^3H]P, i.e., peritoneum or lung, retained only traces of unmetabolized [^3H]P.

With respect to the organs of the pups, P was metabolized extensively to the two pregnanediols, 5β-pregnane-3α,20β-diol and 5α-pregnane-3β,20β-diol, in liver. Both the ^3H- and ^{14}C-labeled 5β-isomers were present in larger amounts than were the ^3H]- and ^{14}C-labeled 5α-isomers. These pregnanediols were identified also as relatively significant metabolites of P in placenta. The other major metabolites of P were the pregnanolones, 3α-hydroxy-5β-pregnan-20-one and 3β-hydroxy-5α-pregnan-20-one. These were the predominant metabolites in organs other than liver. The maternal uterus and the terminal perfusate contained at least trace amounts of all of the identified metabolites listed in Tables I and II.

Discussion

The compounds reported represent a selective search for expected major C_{21} metabolites of P in order to determine both the functioning of the perfusion preparation and the general pattern of metabolism. Fetal organs of significance with respect to steroid metabolism not analyzed in detail included the adrenal glands and gonads. While these organs contained relatively large amounts of ^3H-labeled material per gram of tissue, the amount per organ was insufficient to permit analysis. Very small amounts of ^{14}C-labeled material were detected also in the adrenals and gonads.

Generally, the same compounds were found in all organs investigated, and both [^3H]P and [^{14}C]P were converted to the same metabolites. There were differences among the organs with respect to the amounts of individual compounds found relative to other compounds. The only important qualitative difference between the metabolism of P in the human and in our canine gravid uterine preparation was the finding of large amounts of 5β-pregnane-3α,20β-diol and 5α-pregnane-3β,20β-diol rather than 5β-pregnane-3α,20α-diol, the major urinary metabolite of P during human pregnancy (11). 5β-Pregnane-3α,20α-diol was detected only

in the canine terminal perfusate and, there, only in trace amounts. It is also notable that, perhaps characteristic of the dog, 5α-reduction of P occurred to a fairly great extent. Significant amounts of 5α-pregnanolones were detected, particularly in placenta, intestine, lung, brain, and maternal uterus. Just trace amounts of 20α-hydroxy-4-pregnen-3-one and 20β-hydroxy-4-pregnen-3-one were detected in placentas and injected liver and none in uninjected liver or terminal perfusate. Large amounts of pregnanediols were identified, especially in liver. These findings would seem to indicate both the extensive metabolism of P and the importance of the reductive pathway. However, the dynamics of interconversion of metabolites during the time period of the experiments cannot be evaluated in this model.

A similar preparation of a canine "uterine-placental-fetal preparation *in situ*" was described by Benzi *et al.* (12). These investigators used their preparation to study the metabolism of aminopyrine and to evaluate the distribution in fetal tissues. We have used our preparation also to study the metabolism of [7α-^{3}H]- and [4-^{14}C]pregnenolone (unpublished observation). This study, although less detailed than that reported here for P, yielded similar results. P and further reduced C_{21} metabolites were found in all fetal organs analyzed and in the maternal uterus with one exception. As found in the P perfusion experiments, no P could be detected in uninjected liver. The percentage of unmetabolized pregnenolone detected in the organs was three to four times greater than the percentage of unmetabolized P detected in the P perfusion experiments.

The isolated canine gravid uterus did metabolize P extensively and the products identified were metabolites consistently found in other investigations. ^{3}H- and ^{14}C-labeled compounds appeared to more back and forth, readily crossing the placenta and, ultimately, being distributed in identical fractions. The sites of metabolism are not known, but at the end of 1 hr there was complete mixing. The compounds were the same in fetal and maternal organs. Separate fetal and maternal metabolism could not be distinguished. Either the compounds entered some common metabolic pool and perhaps were metabolized further by the maternal uterus, or the same metabolism took place on each side of the placenta. Therefore, what is outlined is the metabolism of P in the utero-placental-fetal unit. This canine unit, isolated by perfusion of the gravid uterus, has been shown to be a fruitful experimental construct and could be employed under varied conditions as a model to study steroid metabolism.

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