Progesterone metabolism during embryonic diapause in the tammar wallaby, *Macropus eugenii**

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Summary. Metabolic clearance rate (MCR), production rate (PR) and plasma protein-binding of progesterone were measured during embryonic diapause and after ovariectomy in the tammar wallaby. The effect of ovine prolactin and LH on the secretion of progesterone by the quiescent corpus luteum *in vitro* was also examined.

During embryonic diapause, a low plasma progesterone concentration of 196 ± 24 (s.e.m.) pg/ml ($n = 7$) was associated with a low PR of 20.2 ± 1.4 ng/kg/min and a high MCR of 111.0 ± 7.6 ml/kg/min. Similar values were obtained for the 4 animals ovariectomized 9 or 12 months previously (plasma progesterone = 156 ± 14 pg/ml; MCR = 147 ± 16 ml/kg/min; PR = 20.4 ± 2.2 ng/kg/min) although plasma progesterone concentration was decreased by about 50% within 14 days of ovariectomy. An increased adrenal cortical secretion of progesterone in long-term ovariectomized tammar is suggested.

Progesterone was bound by plasma protein. The association constant at 20°C was similar for intact ($2.31 \times 10^8 \text{ M}^{-1}$) and ovariectomized ($2.27 \times 10^8 \text{ M}^{-1}$) tammar while the concentration of binding sites was significantly lower in ovariectomized ($1.41 \times 10^{-7} \text{ M}$) than in intact ($2.28 \times 10^{-7} \text{ M}$) tammar. Cortisol and 17α -hydroxyprogesterone competed with progesterone for binding sites while 20α -dihydroprogesterone, oestradiol- 17β and testosterone did not.

Progesterone was secreted by quiescent corpora lutea *in vitro* but neither LH nor prolactin changed its rate of secretion.

Introduction

The female tammar wallaby (*Macropus eugenii*) mates and usually conceives within a day of parturition, as do most other members of the Family Macropodidae (see Tyndale-Biscoe, 1973; Tyndale-Biscoe, Hearn & Renfree, 1974). The new pregnancy does not proceed to term but is arrested, together with the corpus luteum (CL), at the blastocyst stage and normally remains so until the next summer solstice, some 11 months later (Berger, 1966). This condition, termed quiescence, may be terminated in the early part of the annual cycle by removal or loss of the sucking pouch young (Renfree & Tyndale-Biscoe, 1973), or by the suppression of prolactin secretion with bromocriptine (C. H. Tyndale-Biscoe & L. Hinds, unpublished observations). Development also resumes after hypophysectomy (Hearn, 1973), unless followed by prolactin treatment when quiescence continues for the duration of treatment (Tyndale-Biscoe & Hawkins, 1977). These experiments provide evidence for an inhibitory role of prolactin in embryonic diapause and the recent finding of luteal prolactin-specific binding sites (Sernia & Tyndale-

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Biscoe, 1979) indicates that the CL is probably a major target tissue. Unlike the luteotrophic function of prolactin in the rat, ferret and sheep (Donovan, 1963; Denamur, Martinet & Short, 1973; Ford & Yoshinaga, 1975), in the tammar wallaby prolactin may suppress progesterone secretion by the CL and in this way prevent the induction of uterine conditions required for continued growth of the conceptus (Renfree & Tyndale-Biscoe, 1973).

However, relatively little is known of the steroidogenic function of the CL in this species. Progesterone is synthesized *in vitro* and has been measured in extracts of quiescent and reactivated CL (Renfree & Heap, 1977; Renfree, Green & Young, 1979). Plasma concentrations of progesterone during the oestrous cycle and gestation have been reported (Lemon, 1972; Renfree & Heap, 1977), but there is no detailed information on progesterone metabolism *in vivo* during various reproductive states and, in particular, during embryonic diapause. The steroidogenic function of the tammar CL *in vivo* was therefore examined by measuring production rates and metabolic clearance rates of progesterone during embryonic diapause and after ovariectomy. The effect of prolactin on the release of progesterone by CL *in vitro* was also investigated.

Materials and Methods

Tammar wallabies (3.8–5.6 kg) were obtained from a captive colony housed in outdoor grassed pens with access to an oat/lucerne feed mix.

Production and metabolic clearance rate measurements

The production rate (PR) and metabolic clearance rate (MCR) of progesterone were measured by the constant rate infusion method of Tait & Burstein (1964). The animals were anaesthetized with sodium amytal (Surital: Parke-Davies) and a catheter was inserted into the exterior jugular vein of each and exteriorized at the back of the neck, immediately above the scapulae. After one or more days of post-operative recovery a second catheter was inserted into a tail vein and a blood sample was taken (8 ml); the catheter was secured with tape and left in position. The tammars were accommodated in hessian bags with suitable slits for the tail and jugular catheters. Within the darkness of the bag, and in a quiet environment, these normally excitable animals became relatively docile and further restraint was unnecessary.

After a short period of acclimatization, 10 μCi [^3H]progesterone in 3 ml 10% (v/v) ethanolic saline were quickly injected via the jugular catheter and 10 min later a constant infusion of 2–3 $\mu\text{Ci/h/kg}$ was begun using a motor-driven pump (B. Braun, West Germany). The specific activity of the plasma reached a steady state within 2 h of the start of infusion and thereafter 3 blood samples (6–10 ml) were collected via the tail vein at 20-min intervals. Plasma was separated from erythrocytes by centrifugation at room temperature and plasma progesterone was extracted by 3×5 volumes of redistilled hexane. The extract was evaporated to dryness by a stream of air and the residue was redissolved in 3 ml hexane. The equivalent of 1 ml plasma was assayed for progesterone and 5 μg authentic radioinert progesterone were added to the remaining extract. The [^3H]progesterone and the authentic carrier were purified by thin-layer chromatography using a developing system of cyclohexane/iso-octane/chloroform (22/25/30 by vol.) and the recovered radioactivity was measured by liquid scintillation counting. Procedural losses of $30.0 \pm 1.8\%$ (s.e.m.) ($n = 5$) were calculated from plasma samples containing known amounts of [^3H]progesterone.

Binding of progesterone to plasma protein

Endogenous steroids were removed from plasma by treatment with charcoal (Norit A; 50 mg/ml) for 1.5 h at room temperature. To a sample (100 μl) of this plasma was added

100 μ l sodium phosphate buffer (10 mM, pH 7.6) containing 70 000 d.p.m. [3 H]progesterone and radioinert progesterone, 17 α -hydroxyprogesterone, 20 α -dihydroprogesterone, cortisol, oestradiol-17 β or testosterone (Sigma). After incubation for 12 h at 2°C, the sample was transferred to a 0.7 $\times$ 18 cm column of Sephadex G-25F (Pharmacia) and eluted at 2°C with 10 mM-phosphate buffer (pH 7.6). Fractions of 0.3 ml were collected and the radioactivity was measured. Data for the estimation of association constant and concentration of progesterone binding sites were obtained by the microcolumn method of Ginsburg, Greenstein, MacLusky, Morris & Thomas (1974). Steroid-free plasma was diluted 1:14 with phosphate buffer (10 mM, pH 7.6) containing [3 H]progesterone (70 000 d.p.m.) and various concentrations of non-radioactive progesterone to a final volume of 250 μ l. After a 12 h incubation at 2°C, protein-bound and free progesterone were separated on microcolumns of Sephadex LH-20 at 2°C and the association constant and binding site concentration were estimated from a Scatchard plot of the data (Scatchard, 1949). All data were corrected for non-specific binding, defined as the radioactivity bound in the presence of excess (5×10^{-7} M) progesterone. The concentration of protein in undiluted plasma was measured by the method of Lowry, Rosebrough, Farr & Randall (1951).

CL incubations

Quiescent corpora lutea were obtained from lactating tammar with pouch young (6.3–73.9 g) during late April–early May. All animals were treated with a single i.m. injection of 5 mg bromocriptine (CB154; Sandoz, Australia)/kg and the pouch young were removed approximately 16 h before the parent was killed by stunning and cervical dislocation. A blood sample for later assay of progesterone was taken by cardiac puncture immediately after death. In 3 separate experiments, the single CL from each tammar wallaby was bisected and each half allocated according to a balanced incomplete block design (John & Quenouille, 1977) to a control incubation in buffer alone or a treatment incubation in which the medium contained ovine prolactin (2 μ g/ml: NIH–S10) or ovine LH (2 μ g/ml: NIH–S19). After incubation for 4 h at 35°C (the average body temperature of the tammar) in an atmosphere of 95% O₂–5% CO₂, the CL tissue was removed and the progesterone released into the medium was extracted with 2 $\times$ 10 ml hexane and stored for later radioimmunoassay. The incubation medium in each vial consisted of 4 ml Krebs–Henseleit buffer at pH 7.4 (118 mM-NaCl, 4.7 mM-KCl, 1.4 mM-MgCl₂ · 6H₂O, 2.5 mM-CaCl₂, 1.2 mM-KH₂PO₄, 25 mM-NaHCO₃, 10 mM-glucose).

Progesterone assay

Antiserum to progesterone (Lot S230) was supplied by Dr R. I. Cox, CSIRO, Division of Animal Production Prospect, New South Wales. Antibodies were raised in sheep to progesterone-11-hemisuccinate–BSA. Cross-reactivity relative to progesterone was <0.3% for 17 α -hydroxyprogesterone; 20 α - or 20 β -hydroxy-4-pregnene-3-one; 3 β ,17-dihydroxy-5-pregnene-20-one; 5 β -pregnane-3 α ,20 β -diol; 5 β -pregnane-3 α ,20 α -diol; cortisol and corticosterone. Higher cross-reactivity was present with 11-deoxycorticosterone (1.0%), pregnenolone (1.6%), 5 β -pregnane-3,20-dione (3.5%) and 11 β -hydroxyprogesterone (6.7%).

The assay procedure was based on that of Abraham, Swerdloff, Tulchinsky & Odell (1971). Progesterone in plasma or CL incubation media was extracted with 2 $\times$ 10 volumes of freshly redistilled n-hexane. The pooled extract was cooled in solid CO₂/ethanol to solidify the aqueous phase, and the organic phase was then decanted and dried under air at 37°C. To these dried extracts and to progesterone standards ranging from 10 to 500 pg/tube was added 1.2 ml of assay buffer (1.0% gelatin in 0.2 M-phosphate buffer, pH 7.0) followed by an equilibration period of 4 h at room temperature. Duplicate 0.5 ml aliquots were then pipetted into assay tubes and antiserum (1:1000 dilution) and 10 000 c.p.m. [1,2,6,7(N)- 3 H]progesterone (Lot

1004-110, sp act. 114 Ci/mmol: New England Nuclear) were added in a 0.2 ml volume, with thorough mixing of contents. After a 14–18 h incubation at 4°C free steroid was removed by charcoal treatment (0.2 ml of 0.625% in assay buffer) for 30 min. The charcoal was sedimented by centrifugation (2500 g, 15 min) and 0.5 ml supernatant was added to 10 ml scintillation fluid (42 ml Liquifluor and 150 ml dioxan in 1 litre toluene). After a 4 h extraction period at 4°C, the radioactivity in these samples was measured in a spectrophotometer (Isocap/300, Searle) at an efficiency of 65%. With this procedure, there was no detectable interference from solvents or extracts of charcoal-treated plasma, provided glassware was rinsed in ethanol and the n-hexane redistilled. Efficiency of the extraction procedure was 85–95% as determined from the recovery of radioactive progesterone equilibrated with endogenous plasma progesterone. Assay sensitivity, defined as the least measurable amount of progesterone, was 25 pg/ml. Intra- and inter-assay co-efficients of variation of 9.0 and 13.0% respectively were determined from repeated measures of a single pool of plasma within the same assay ($n = 10$) and in separate assays ($n = 29$). Accuracy was determined by the addition of progesterone to charcoal-treated plasma to a final concentration of 100, 200, 300 or 400 pg/ml. The measured concentration was always within 16% of that expected; a highly significant ($r^2 = 0.996$, $P < 0.001$) linear correlation was present.

Statistics

All data are represented as mean $\pm$ s.e.m. and were analysed by parametric tests except for those in Table 1.

Table 1. Plasma concentration, production rate (PR) and metabolic clearance (MCR) of progesterone in intact (Nos 1–7) and ovariectomized (Nos 8–11) tammar wallabies

Tammar	Body wt (kg)	Progesterone conc. (pg/ml)		MCR (ml/kg/min)	PR (ng/kg/min)
		Pre-infusion	Steady state		
Intact					
1	4.44	103	157	100	15.7
2	3.92	119	187	126	23.6
3	4.42	97	167	124	20.7
4	4.04	122	172	121	20.8
5	4.93	461	331	70	23.2
6	4.24	157	132	109	14.4
7	4.42	140	181	124	23.1
Mean $\pm$ s.e.m.	4.34 $\pm$ 0.12	171 $\pm$ 49	196 $\pm$ 24	111 $\pm$ 7.6	20.2 $\pm$ 1.4
Ovariectomized					
8*	4.68	337	128	153	19.6
9†	4.06	131	147	179	26.3
10*	4.40	81	102	155	15.8
11†	4.05	149	193	102	19.7
Mean $\pm$ s.e.m.	4.30 $\pm$ 0.15	175 $\pm$ 56	156 $\pm$ 14	147 $\pm$ 16	20.4 $\pm$ 2.2

* Ovariectomized for 9 months.

† Ovariectomized for 24 months.

Results

Production and metabolic clearance rates

These values were measured in 7 post-lactational females with quiescent corpora lutea and in 4 females ovariectomized 9 or 24 months previously. All measurements were made in November, approximately 2 months before the annual breeding season.

The concentration of progesterone during the pre-infusion period was comparable to that during steady-state conditions ($P > 0.05$, Wilcoxon's matched pairs test), although a greater variation was present in the pre-infusion samples (Table 1). In steady-state conditions, the plasma concentration and MCR of progesterone in ovariectomized tammar were surprisingly similar to those of intact tammar; the production rate for both groups was identical. There was some suggestion of an inverse relationship between MCR and plasma progesterone concentration but non-parametric tests indicated a poor correlation (Spearman's $r = 0.58$, $P < 0.1$).

In a further 5 tammar wallabies in seasonal quiescence the short-term changes in plasma progesterone concentration following bilateral ovariectomy were measured. The concentration decreased from 206 ± 8 pg/ml before surgery to 141 ± 9 and 99 ± 6 pg/ml at 7 and 14 days after surgery respectively ($P < 0.01$, analysis of variance for repeated measures).

Binding to plasma proteins

Chromatography showed that most of the progesterone was bound to protein (Text-fig. 1a). Incubation in the presence of 1×10^{-7} M-radioinert progesterone (data not shown), cortisol or 17α -hydroxyprogesterone decreased the protein-bound [3 H]progesterone by 90, 65 and 17% respectively, while 20α -dihydroprogesterone, oestradiol- 17β and testosterone were ineffective ($< 3\%$) in displacing bound [3 H]progesterone.

Text-fig. 1. Steroid-binding properties of tammar wallaby plasma. (a) Separation at 2°C of protein-bound and unbound [3 H]progesterone (—) on a column of Sephadex G-25 and displacement by non-radioactive 17α -hydroxyprogesterone (.....) and cortisol (---). (b) A Scatchard plot of the binding of progesterone to diluted (1:14 v/v) tammar wallaby plasma ($K_a = 2.43 \times 10^8 \text{ M}^{-1}$; $N = 1.91 \times 10^{-8} \text{ M}$).

The Scatchard analysis of the binding data is shown in Text-fig. 1(b). Values for the association constant fell in a narrow range (Table 2) with no significant difference between intact and ovariectomized tammar, but there were significantly more binding sites in the intact than in the ovariectomized animals. The plasma protein concentration was also lower in the ovariectomized tammar. A regression analysis of the data from all 11 animals indicated a significant correlation ($r = 0.75$, $P < 0.05$) between protein concentration and progesterone binding sites.

Secretion of progesterone in vitro

The amounts of progesterone secreted by the luteal tissue in the 3 experiments are shown in Table 3. An analysis of variance of the pooled data indicated that there were no differences

Table 2. Association constant and concentration of binding sites for the binding of progesterone to plasma proteins of intact (Nos 1–7) and ovariectomized (Nos 8–11) tammar wallabies

Tammar	Protein conc. (g/100 ml plasma)	Association constant ($\times 10^{-8} \text{ M}^{-1}$)	Binding site conc. ($\times 10^7 \text{ M}$)
Intact			
1	6.69	2.33	1.76
2	6.69	2.35	2.52
3	6.77	2.60	2.30
4	6.16	2.36	1.84
5	7.11	2.02	2.05
6	6.80	2.12	2.67
7	7.26	2.43	2.86
Mean $\pm$ s.e.m.	6.78 $\pm$ 0.13*	2.31 $\pm$ 0.07	2.28 $\pm$ 0.16**
Ovariectomized			
8	6.98	2.32	1.78
9	5.40	2.13	1.49
10	5.51	2.40	0.95
11	5.84	2.23	1.43
Mean $\pm$ s.e.m.	5.93 $\pm$ 0.36*	2.27 $\pm$ 0.06	1.41 $\pm$ 0.17**

* $P = 0.05$, ** $P < 0.01$.**Table 3.** Effect of prolactin and LH on progesterone release *in vitro* by luteal tissue of tammar wallabies in quiescence*

Exp.	No. of animals	Wt of CL (mg)	Plasma progesterone (pg/ml)	Replicates/treatment	Progesterone secretion (ng/mg/4 h)		
					Control	Prolactin (2 $\mu\text{g/ml}$)	LH (2 $\mu\text{g/ml}$)
A (May)	6	11.9 $\pm$ 1.1	186 $\pm$ 21	4	41.3 $\pm$ 0.8	27.1 $\pm$ 3.1	42.2 $\pm$ 0.8
B (May)	6	10.9 $\pm$ 0.6	188 $\pm$ 25	4	20.6 $\pm$ 1.3	49.8 $\pm$ 13.6	41.0 $\pm$ 12.7
C (July)	6	8.4 $\pm$ 0.5†	163 $\pm$ 20	4	46.2 $\pm$ 6.0	51.5 $\pm$ 6.6	42.7 $\pm$ 9.2
Overall mean					36.1 $\pm$ 3.8	42.8 $\pm$ 5.7	42.0 $\pm$ 4.7

Values are mean $\pm$ s.e.m.

* Endogenous prolactin secretion was suppressed by an injection of bromocriptine (5 mg/kg, i.m.) and removal of sucking pouch young 16 h before the experiment.

† Significantly different from values in Exps A and B ($P < 0.05$).

between the 3 treatment groups, although there was a difference between the experiments. Progesterone concentration and CL weight were lower in Exp. C but only the latter was significantly so. The correlation between plasma progesterone and weight of corpus luteum in all 18 animals was poor ($r = 0.29$, $P > 0.1$).

Recent corpora albicantia from tammar with quiescent corpora lutea were also harvested and incubated without pituitary hormones in conditions identical to those for the corpus luteum. The progesterone released over a 4 h period was 7.2 ± 1.3 ng/mg.

Discussion

Unusually low plasma concentrations of progesterone during the oestrous cycle and pregnancy in the tammar wallaby were first reported by Lemon (1972) who measured the steroid with a protein-binding assay. Her report has since been supported by Renfree & Heap (1977) and is

also confirmed in the present investigation in which a sensitive and specific radioimmunoassay was used. Other marsupials investigated so far do not have such low concentrations of progesterone and therefore the low values in the tammar wallaby are unlikely to represent a common feature of marsupials (Cook & Nalbandov, 1968; Thorburn, Cox & Shorey, 1971; Gaughwin & Wells, 1978).

The plasma concentration of progesterone is a function of the rate of production by ovarian and, to a lesser extent, adrenal tissue, and of the rate of clearance by hepatic and other tissues. When compared to several other mammalian species for which data are available (Table 4), it becomes evident that the low plasma concentrations of progesterone in the tammar wallaby are due to a high metabolic clearance rate and a low production rate; the latter being comparable to the rate in ovariectomized eutherian mammals. However, this low secretory activity does not indicate that progesterone is dispensable during embryonic diapause, because ovarian progesterone is important for the long-term viability of dormant blastocysts (Tyndale-Biscoe, 1974). Higher concentrations of this steroid, as after injection, appear to signal the resumption of embryonic development (Renfree & Tyndale-Biscoe, 1973). The low secretory activity during embryonic diapause may therefore be part of an adaptational mechanism by which progesterone maintains embryonic viability without interrupting diapause.

Table 4. Metabolic clearance rate (MCR) and production rate (PR) of progesterone in various mammalian species

Species	Reproductive state	MCR* (l/day/kg)	PR* (mg/day)	Reference
Rat	Ovariectomized	103	0.13	Pepe & Rothchild (1973)
	Pregnant (Day 15)	112	0.46	
Rabbit	Non-pregnant	230	0.5	Thau & Lanman (1975)
Rhesus monkey	Follicular phase	50	<0.15	Thau <i>et al.</i> (1977)
	Luteal phase	55	1.9	
Baboon	Non-pregnant	53.1	4.57	Albrecht & Townsley (1976)
	Pregnant	87.3	15.26	
Man	Ovariectomized	32.8	0.8	Little <i>et al.</i> (1966)
	Pregnant, 3rd trimester	31.1	210	Lin <i>et al.</i> (1972)
Tammar wallaby	Ovariectomized	212	0.13	Present study
	During embryonic diapause	160	0.13	

* Values were calculated from published data for conscious animals. Insufficient details of individual animals prevented the calculation of PR as a function of body weight.

The similar production rates of progesterone in intact and long-term ovariectomized tammar were surprising because, in a separate group, progesterone concentrations had been reduced to 50% by 14 days after ovariectomy. A compensatory mechanism must presumably be acting in ovariectomized tammar; the eventual increased progesterone production probably being caused by increased secretion from the adrenal cortex.

Corticosteroid-binding and sex-hormone-binding proteins analogous to CBG and SHBG of eutherian mammals have been characterized in numerous marsupial species, including the tammar (Sernia, Bradley & McDonald, 1979). Marsupial CBG is reported to have a high affinity for both cortisol and progesterone, although the affinity for progesterone was not quantitated. In the present investigation a high-affinity progesterone-binding system was found with a capacity similar to that measured for cortisol by Sernia *et al.* (1979). Furthermore, bound [³H]progesterone was displaced only by cortisol and to a minor extent by 17 α -hydroxyprogesterone, but not by 20 α -dihydroprogesterone, testosterone or oestradiol-17 β . These data suggest that this binding protein has ligand-binding properties like eutherian CBG (see Westphal, 1971) and that progesterone is not bound to a SHBG-like protein, which is in accord with the

reported absence of SHBG in the tammar (Sernia *et al.*, 1979). The concentration of progesterone-binding sites of about 300 times that of progesterone during embryonic diapause implies that the plasma steroid is largely in a bound form. Since the protein-bound form is thought to be metabolically inert (Slaunwhite, Lockie, Black & Sandberg, 1962), the higher MCR of ovariectomized tammars may be due to a significantly lower concentration of CBG (Table 2), probably resulting from the loss of ovarian oestrogens (see Westphal, 1971).

In eutherian mammals, LH stimulates progesterone secretion *in vivo* and *in vitro* (Armstrong, 1968) while prolactin, a luteotrophic hormone in several species, does not (Ford & Yoshinaga, 1975). Similarly, progesterone secretion by the luteinized ovaries of the American opossum (*Didelphis*) is stimulated by ovine LH but not by FSH or prolactin (Cook & Nalbandov, 1968). In the present investigation, isolated luteal tissue released about 4 times its reported progesterone content (Renfree *et al.*, 1979) but neither ovine LH nor prolactin had any effect on progesterone secretion. These results cannot be explained simply as non-recognition of ovine hormones by marsupial tissues since both ovine LH and prolactin bind specifically to marsupial gonadal tissues (Bona-Gallo, Licht, Farmer, Papkoff & Hawkins, 1978; Sernia & Tyndale-Biscoe, 1979). The failure of ovine LH to stimulate progesterone secretion in the tammar is, however, consistent with the observation that reactivation occurs after hypophysectomy, i.e. in the complete absence of LH (Hearn, 1973), and further supports the notion that the quiescent corpus luteum is not stimulated by LH.

On the basis of previous reports (Hearn, 1973; Tyndale-Biscoe & Hawkins, 1977), prolactin was expected to have inhibited progesterone secretion by the tammar CL *in vitro*. The failure to observe such an effect probably indicates either that repression of steroidogenesis in the quiescent CL is already maximal or that steroidogenesis may not be the primary site of action of prolactin. The role of pituitary hormones in luteal function in the tammar wallaby is being further investigated.

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