PROGESTERONE CONCENTRATIONS IN PERIPHERAL PLASMA
OF NON-PREGNANT AND PREGNANT GREY SQUIRRELS
(SCIURUS CAROLINENSIS)

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SUMMARY

Plasma concentrations of progesterone in non-pregnant female grey squirrels were never
greater than 3.2 nmol/l and no significant differences were found between levels in
anoestrous, pro-oestrous and oestrous animals. During pregnancy, plasma concentrations of
progesterone increased significantly and reached a maximum level of 318 nmol/l at around
day 35 of the 44 day period of gestation. After parturition, plasma concentrations of
progesterone fell sharply. The corpora lutea of pregnancy began to regress in size at about day
30 of gestation, before the maximum levels of progesterone in the plasma were reached, which
suggests that there is an extra-ovarian source of progesterone.

Chromatography of pregnancy plasma extracts showed that no significant amount of 5α-
or 5β-pregnane-3,20-dione was present and that progesterone accounted for 90% of the
assay-positive material in pregnancy plasma from grey squirrels.

INTRODUCTION

The American grey squirrel (Sciurus carolinensis) was introduced into Britain from the
U.S.A. towards the end of the nineteenth century, since when it has spread throughout
England, Wales and parts of Scotland. In both Britain and the U.S.A. pregnant females have
been captured in all months from January to August, although peaks in litter production are
usually seen in March/April (spring) and June/July (Summer) (Deanesly & Parkes, 1933;
Nixon & McClain, 1975). In the non-breeding season female squirrels are anoestrous, with
ovaries containing small Graafian follicles (400–600 µm diameter). The uterus is ribbon-like
and the vulva is small and inconspicuous. With the approach of oestrus the Graafian follicles
enlarge, the uterus becomes large and turgid and the vulva swollen (Deanesly & Parkes,
1933). Mating seems to occur soon after the female has entered oestrus (Thompson, 1977) but
it is not known whether ovulation is spontaneous or induced by copulation.

In the present study plasma concentrations of progesterone in both non-pregnant and
pregnant female grey squirrels have been measured by radiomimunoassay. Non-pregnant
squirrels were examined in order to determine whether plasma concentrations of pro-
gersterone rose at oestrus as seen in a number of species such as the rat (Cortes, McCracken,
Lloyd & Weisz, 1971), guinea-pig (Feder, Resko & Goy, 1968), sheep (Wheeler, Baird, Land
& Scaramuzzi, 1975) and hamster (Hoffman & Fajer, 1970). Pregnant squirrels were
examined in order to elucidate the pattern of changes in plasma concentrations of
progesterone during pregnancy and to examine the sites of progesterone production.

Since captive female grey squirrels do not maintain regular sexual activity it was necessary
to carry out the investigations entirely on squirrels caught in the wild.
Squirrels were trapped alive in Legg Multicatch traps (Fuller Engineering Ltd, Worthing, Sussex) at monthly intervals over 2 years in an area of enclosed woodland managed by the Forestry Commission in southern England (National grid ref: SU 806 416). The traps, which allow the entry of squirrels but not their exit, have a large holding compartment in which the squirrels are retained unharmed. Of the 94 female squirrels caught, 67 non-pregnant and 22 pregnant females were killed with CO₂ and examined a few hours after capture. The remaining five squirrels, all in the early stages of pregnancy, were maintained in captivity to allow serial blood samples to be taken during gestation. Three of these animals produced litters, and the remaining two animals died before delivery, one at day 10–11 of gestation and the other at day 40–42 of gestation.

The five squirrels maintained in captivity were housed individually on the roof of a seven-storey building in weldmesh cages containing a wooden nesting box. The animals received an unrestricted amount of pelleted Diet 86 (Dixon & Sons Ltd, Ware, Herts.) and had constant access to drinking water. They were exposed to natural climatic conditions except for a cover to protect them from the rain.

The age of each animal killed after capture was established using the dried eye-lens weight together with the degree of fusion of the epiphyses of the wrist (Dubock, 1979); all the non-pregnant animals used were adults (more than 15 months old).

A vaginal smear was taken from all animals immediately before killing in order to determine the stage of the oestrous cycle (Deanesly & Parkes, 1933). Smears were taken on the day of capture from those pregnant animals maintained in captivity and on each occasion when a blood sample was collected.

The stage of gestation at which pregnant squirrels were killed and at which the two captive squirrels died was determined using the crown–rump length of the fetuses together with the size of the uterine swellings as described by Smith (1967).

**Histology**

Ovaries were fixed in alcoholic Bouin’s solution, embedded in Fibrowax (Raymond A. Lamb, London), serially sectioned at 8 μm and stained with haematoxylin and eosin. In non-pregnant squirrels the number of non-atretic Graafian follicles was recorded and the size of each obtained by taking the mean of two diameters, measured at right angles, in the largest section of the follicle. In pregnant squirrels the number of corpora lutea in each ovary was counted and the volume (V) of each was calculated using the formula: \( V = (\pi/6) d_1 \cdot d_2 \cdot d_3 \), where \( d_1 \) is the product of the number of sections in which the corpus luteum appears and the section thickness (8 μm), and \( d_2 \) and \( d_3 \) are the two diameters (μm) measured at right angles in the largest section of the corpus luteum (Rowlands, 1961).

**Blood collection**

At autopsy blood was collected directly from the heart using a 10 ml heparinized syringe. After centrifugation plasma was stored at −20 °C. Blood samples from the live, captive, pregnant squirrels were collected between 09.00 and 12.00 h at approximately weekly intervals. The animals were restrained in a weldmesh cylinder with their heads covered and blood was collected without anaesthesia by cutting off the tip (1 mm) of the tail and allowing 1.0–1.5 ml to drip into a heparinized centrifuge tube, after which the wound was sealed using Nobecutane spray (B.D.H., London). After centrifugation the plasma was stored at −20 °C.
Plasma progesterone levels in the grey squirrel

For the three animals which gave birth, the time during gestation at which blood samples were taken was estimated by calculation from the day of parturition which was assumed to be day 44 (Asdell, 1964; Smith, 1967). For the two squirrels which died during gestation the time of blood collection was calculated from the estimated stage of gestation at death.

Radioimmunoassay for progesterone

The antiserum, G 465/7, used in this study, was raised in a goat against 11α-hydroxyprogesterone–hemisuccinate–bovine serum albumin conjugate (Furr, 1973). The method used for the measurement of plasma concentrations of progesterone was that of Glencross, Munro, Senior & Pope (1973) with minor modifications. [1, 2, 6, 7(n)-3H]Progesterone was obtained from The Radiochemical Centre, Amersham, Bucks. For plasma samples of low progesterone concentration, [3H]progesterone (2000 disintegrations/min; 11·1 fmol) and standards of 0, 16, 32, 64, 160, 320, 640 fmol and 20 pmol, each in duplicate, were used and for samples of higher progesterone concentration, [3H]progesterone (5000 disintegrations/min; 27·4 fmol) and standards of 0, 160, 320, 640 fmol and 1·3, 2·6, 5·2, 10·4 and 104 pmol, each in duplicate, were used. Both progesterone standards (Koch Light, Colnbrook, Berks.) and plasma samples (100 or 200 µl) were submitted to the extraction procedure of Glencross et al. (1973). Antiserum G 465/7 was used at final dilutions of 1 : 100 000 and 1 : 12 500 respectively for the two ranges of standards described above. The final volume at the incubation stage was 500 µl, from which 100 µl were removed for estimation of recovery of tracer.

Accuracy was assessed by adding 64, 160 or 320 fmol or 0·65, 1·3 or 2·6 pmol progesterone to 100 µl distilled water. The quantities of progesterone measured were, respectively, 63·6 ± 4·6 fmol (s.e.m.; n = 9), 174·7 ± 10·4 fmol (n = 7), 326·6 ± 13·2 fmol (n = 11), 0·57 ± 0·1 pmol (n = 5), 1·27 ± 0·0 pmol (n = 3) and 2·3 ± 0·3 pmol (n = 5). Intra- and interassay precision (coefficients of variation) were 5·8 and 14·0% assessed at the level of 320 fmol and 13·0 and 20·0% assessed at the level of 640 fmol. The sensitivity of the assay was obtained from the binding inhibition curves using the lower range of standards. The smallest amount of progesterone which could be distinguished from zero at 95% confidence limits was equivalent to a concentration of 0·16 nmol/l. Recovery of [3H]progesterone was 75·6 ± 1·1% (n = 94) from standards and 73·2 ± 0·6% (n = 150) from plasma samples. Correction for manipulative losses was made on the basis of individual recoveries. Recovery of other tritiated steroids, 17α-hydroxy-4-pregnene-3,20-dione, oestrone and corticosterone, from squirrel plasma was 32·7 ± 1·2, 39·2 ± 1·8 and 10·2 ± 0·8% (n = 6) respectively. Using a Comparison of Regressions Program, there was no significant difference between the slopes of the binding inhibition curves and those obtained from increasing amounts of squirrel plasma (P = 0·002). Cross-reactions of the antiserum G 465/7, using the methods described by Furr (1973), were 20% with 5α-pregnan-3,20-dione, 54% with 11α-hydroxy-4-pregnene-3,20-dione and less than 1% with other progestins such as 17α-hydroxy-4-pregnene-3,20-dione and 20α-hydroxy-4-pregnene-3-one (B. E. Senior, personal communication).

Assay of placental tissue

Placental tissue from a squirrel at 37–38 days of gestation was frozen on solid CO2 at autopsy. A weighed sample was homogenized in 0·5 ml distilled water after the addition of 5000 disintegrations/min [3H]progesterone to measure procedural losses and also to act as the labelled ligand in the radioimmunoassay. The homogenate was extracted in the same manner as the plasma samples. The resulting dried extract was re-dissolved in toluene giving a concentration corresponding to 0.01 g wet weight tissue/ml and subjected to the same assay procedure as for plasma samples. There was no significant difference between the slopes of
the binding inhibition curves and those obtained from serial dilutions of placental extract ($P = 0.002$).

**Chromatography**

Sephadex LH-20 (Pharmacia Ltd, Uppsala, Sweden) chromatography was used, first to separate progestins from other steroids which cross-react with the antiserum and secondly to determine whether 5α-pregnane-3,20-dione was present in significant amounts in the plasma of pregnant grey squirrels.

Progesterone was separated from steroids other than pregnane-3,20-dione isomers by chromatography on Sephadex LH-20 using a solvent system of cyclohexane : ethanol (80 : 20, v/v) (Setchell & Shackleton, 1973). The gravity-feed column with a flow rate of 12 ml/h was 37 x 1 cm and was calibrated with four tritiated steroids, progesterone (2 x 10$^6$ disintegrations/min), 17α-hydroxy-4-pregnene-3,20-dione (16 x 10$^5$ disintegrations/min), oestrone (15 x 10$^5$ disintegrations/min) and corticosterone (3 x 10$^6$ disintegrations/min) from The Radiochemical Centre. Fractions of 3 ml were collected. Samples of pregnancy plasma containing 100 000 disintegrations/min of each of the tritiated steroids, progestrone, 17α-hydroxy-4-pregnene-3,20-dione, oestrone and corticosterone, were subjected to the extraction procedure of the progestrone radioimmunoassay and the extracts chromatographed on the Sephadex column. A sample of placental tissue extract was similarly chromatographed.

To separate progesterone and 5α-pregnane-3,20-dione a similar Sephadex LH-20 column (37 x 1 cm) was prepared with 90% aqueous methanol and iso-octane saturated with 90% aqueous methanol (Castracane, Rahman & Billiar, 1975). The column was calibrated in three ways, with [3H]progesterone (10 000 disintegrations/min, 56 fmol) added to 16 pmol unlabelled progesterone (Koch Light), with 16 pmol 5α-pregnane-3,20-dione (Sigma Ltd, Poole, Dorset) and with 16 pmol 5β-pregnane-3,20-dione (Sigma Ltd). Eluted fractions were subjected to the progestrone radioimmunoassay procedure. In addition, radioactivity of the eluted progesterone fractions was counted. Samples of pregnancy plasma containing 10 000 disintegrations/min of tritiated progesterone were subjected to the extraction procedure of the radioimmunoassay and extracts chromatographed on the column.

**Scintillation counting**

Tritium was counted using 5 ml scintillant in a liquid scintillation spectrometer (Packard Instrument Co. Illinois) at an efficiency of 45%. The scintillant contained Butyl PBD (0.6%, w/v; Koch Light) and POPOP (0.01%, w/v; Koch Light) per litre toluene (Fisons Ltd, Loughborough, Leics.).

**RESULTS**

**Vaginal smears**

Non-pregnant squirrels were grouped as anoestrus, pro-oestrous or oestrous on the basis of the vaginal smear. Cell types at each stage corresponded to those described for the rat (Long & Evans, 1922). The smear from squirrels classified as anoestrus was usually devoid of all cells but occasionally small nucleated epithelial cells could be seen. Smears from animals classified as in pro-oestrous consisted of sheets of larger nucleated epithelial cells. Animals classified as in oestrus had smears which were thick and consisted of cornified epithelial cells only. Squirrels caught in early pregnancy always showed a metoestrous-type smear with cornified epithelial cells and many polymorphonuclear leucocytes. Adult female squirrels trapped during the months of August to November were always anoestrous. Over the period of study,
squirrels were caught showing pro-oestrous and oestrous vaginal smears in May, June and July and pregnant squirrels were trapped during the months of January, February, May, June and July.

**Plasma concentrations of progesterone in non-pregnant squirrels**

Table 1 shows the mean diameter of the largest Graafian follicle and the mean and range of plasma concentrations of progesterone in anoestrous, pro-oestrous and oestrous squirrels. There was a significant difference in size of the largest follicle between anoestrous and oestrous animals but mean plasma concentrations of progesterone were not significantly different.

<table>
<thead>
<tr>
<th>Vaginal smear type</th>
<th>No. of animals</th>
<th>Diameter of follicle (μm)</th>
<th>Progesterone (nmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean ± S.E.M.</td>
</tr>
<tr>
<td>Anoestrous</td>
<td>48</td>
<td>535 ± 13</td>
<td>0·80 ± 0·13</td>
</tr>
<tr>
<td>Pro-oestrous</td>
<td>7</td>
<td>588 ± 24</td>
<td>1·27 ± 0·41</td>
</tr>
<tr>
<td>Oestrous</td>
<td>12</td>
<td>687 ± 33*</td>
<td>0·89 ± 0·13</td>
</tr>
</tbody>
</table>

*P < 0·001 compared with anoestrous animals.

**Plasma concentrations of progesterone in pregnant squirrels**

Plasma concentrations of progesterone during pregnancy were much greater than those found in non-pregnant squirrels. The plasma progesterone profiles of three animals which reached term are shown in Fig. 1 (a), (b) and (c). In all three animals a biphasic pattern of progesterone secretion was seen with peaks of 19·1–31·8 nmol/l and 222·6–318·0 nmol/l occurring in the periods of days 5–15 and 30–40 respectively. In two of the squirrels sampled shortly after parturition the progesterone concentrations were 3·7 nmol/l (Fig. 1a) and 1·1 nmol/l (Fig. 1b). The progesterone concentrations in both these animals had fallen to around 0·6 nmol/l a few days later. In the third squirrel (Fig. 1c) progesterone concentrations were high (64 nmol/l) on the day of parturition of two young. However, a third squirrel was born on the following day, after which plasma concentrations of progesterone fell rapidly to around 0·6 nmol/l. There was no relationship between the number of young born and the maximum concentrations of plasma progesterone.

Maximum volume of corpus luteum (range 0·60–1·20 mm3) occurred between days 10 and 30 of gestation. After day 30 the corpora lutea began to decrease in size and at parturition the volume had decreased to around 0·10 mm3. In Fig. 1d the progesterone profile, obtained from the mean results of all five animals held captive during pregnancy, is shown together with the mean volume of corpora lutea. It may be seen that the mean volume of the corpora lutea was decreasing before plasma concentrations of progesterone reached their maximum.

Radioimmunoassay of placental extract at 37–38 days of gestation showed that progesterone was present at a concentration of 3·2 nmol/g placental tissue.

**Chromatography**

Elution of the Sephadex LH-20 column with cyclohexane : ethanol showed [3H]progesterone in fraction 7,17α-hydroxy-4-[3H]pregnene-3,20-dione in fractions 11 and 12, [3H]oestrone in fractions 16 and 17 and [3H]corticosterone in fractions 19–21. Chromatography of the extract of pregnancy plasma to which the four tritiated steroids had been added showed
Fig. 1. (a), (b) and (c) Plasma concentrations of progesterone during pregnancy in three female grey squirrels which produced litters. Each point is the mean of duplicate determinations. (d) The relationship between plasma concentrations of progesterone (○) and volume of corpus luteum (■) during pregnancy. The progesterone profile is based on the results obtained from five captive pregnant squirrels. The gestation period was divided into intervals of 5 days and a mean (± S.E.M.) progesterone value was calculated. The mean volume of corpora lutea during each interval of 5 days are based on measurements from 22 females killed during pregnancy. P, Day of parturition.
three peaks of radioactivity corresponding to progesterone, 17α-hydroxy-4-pregnene-3,20-dione and oestrone. No peak of radioactivity corresponding to corticosterone was seen as the amount of this steroid extracted by light petroleum is very low. A single peak of assay-positive material was recovered which corresponded exactly with the radioactivity peak that was identified as progesterone. Chromatography of placental tissue also showed a single peak of assay-positive material corresponding with the radioactivity peak of progesterone.

Elution of the column with iso-octane saturated with 90% aqueous methanol (Fig. 2) showed that tritiated progesterone was recovered in fractions 36–42 and unlabelled progesterone, detected using the assay procedure, was recovered in fractions 37–41. 5α-Pregnane-3,20-dione and 5β-pregnane-3,20-dione, also detected using the progesterone radioimmunoassay, were recovered in fractions 15–21 and 9–13 respectively. Minor peaks of immunoreactive impurities were also eluted (Fig. 2b, c). Chromatography of the pregnancy plasma extract is shown in Fig. 3. The main peak of radioactivity was in fractions 36–43 and the main peak of assay-positive material was in fractions 37–41; these peaks corresponded to progesterone and accounted for 90% of the total assay-positive material. A minor peak of assay-positive material was recovered in fraction 28; its position did not correspond with those of progesterone or the pregnanediones.

![Graph](image-url)
DIscussion

Satisfactory evaluation of the specificity, accuracy, precision and sensitivity of the radioimmunoassay procedure, used to measure plasma concentrations of progesterone in grey squirrel plasma, has been made, including demonstration of parallelism of binding inhibition curves obtained from progesterone and plasma and tissue extracts.

The time of year when anoestrous and oestrous vaginal smears were obtained, together with the low concentrations of plasma progesterone found in all non-pregnant squirrels, confirms the hypothesis that the female of this species has intermittent periods of breeding activity. It has been proposed that the pre-ovulatory rise in progesterone, which occurs in a number of spontaneously ovulating species, may facilitate the positive feedback of oestradiol-17β in promoting the sharp rise in luteinizing hormone responsible for ovulation (Harris & Naftolin, 1970). Induced ovulators such as the hare (Stavy, Terkel & Kohen, 1978), cat (Verhage, Beamer & Brenner, 1976) and ferret (Heap & Hammond, 1974) show no such rise in plasma concentrations of progesterone. The low concentrations of plasma progesterone in the grey squirrel except during pregnancy cannot be taken as firm evidence of induced ovulation because of the infrequency of blood sampling. It is hoped that studies of squirrels held in forest enclosures will allow this question to be resolved.

In a number of mammalian species it has been shown that significant amounts of 5α-pregnanedione (5α-pregnane-3,20-dione) are present in the plasma during pregnancy. In the mare this steroid is present in concentrations similar to those of progesterone during early pregnancy and at a concentration greater than that of progesterone during late pregnancy (Atkins, Harms, Sorensen & Fleeger, 1976); in the pregnant woman and rhesus monkey 5α-pregnanedione is present at a concentration of about one-third that of progesterone (Stoa & Bessesen, 1975; Sholl, Robinson & Wolf, 1979). 5α-Pregnanedione is also present in the plasma of the pseudopregnant rat, at a concentration of about half that of progesterone (Shore, 1976). Since the antiserum used in the present study has a significant cross-reaction with 5α-pregnanedione, it seemed desirable to test for the presence of this steroid in plasma from pregnant grey squirrels. Although two peaks of assay-positive material were identified after chromatography of pregnant squirrel plasma, neither corresponded to 5α-pregnanedione or 5β-pregnanedione. The larger peak, which represented 90% of the assay-positive material was identical in its position to progesterone, but the minor peak remains
unidentified. The results show that 5α-pregnanedione is not present in significant amounts in pregnant grey squirrel plasma but that progesterone is present in high concentrations.

The present study has confirmed previous observations that in squirrels the corpora lutea of pregnancy regress before parturition (Deanesly & Parkes, 1933; Dubock, 1976). Regression of the corpora lutea begins at about day 30, before maximum concentrations of plasma progesterone are attained, suggesting an extra-ovarian source of progesterone. The most likely source is the placenta, extracts of which obtained around days 37–38 of gestation had high concentrations of a compound responding in the radioimmunoassay and having the mobility of progesterone in the cyclohexane : ethanol system. Further evidence for the placental source of progesterone was provided by one squirrel which gave birth to two young whilst having a plasma progesterone titre of 64 nmol/l. This was followed by the birth of a third young coincident with a rapid fall in progesterone level.

The pattern of plasma progesterone during pregnancy is similar to that of the musk shrew (Hasler & Nalbandov, 1978) in which the peak in plasma progesterone appears to be of placental origin. The maximum concentrations of progesterone in the squirrel are much higher than in the musk shrew, similar to the concentrations in the rat (Bartholomeusz, Bruce, Martin & Hartmann, 1976) and the mink (Møller, 1973) but considerably lower than those reported for the guinea-pig (Challis, Heap & Illingworth, 1971) and cuis (Tam, 1973). In the musk shrew the dependence on placental progesterone was confirmed by the demonstration that pregnancy continues in the absence of the ovaries after day 5 of gestation. This crucial investigation has not yet been performed in the grey squirrel.

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REFERENCES


