PREGNENOLONE AND PROGESTERONE CONCENTRATIONS IN OVARIAN VENOUS BLOOD OF THE ARTIFICIALLY OVULATED SQUIRREL MONKEY (*SAIMIRI SCIUREUS*)

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Summary. Ovulation was induced in sexually immature squirrel monkeys by PMSG–HCG injections and pregnenolone and progesterone concentrations were determined in the ovarian venous blood. Extensive follicular growth does not permit the diagnosis of ovulation without histological examination of the ovaries. Ovulation was followed by extensive luteinization and detectable amounts of pregnenolone and progesterone 2 days after treatment but, by 5 days after treatment, only five out of eleven animals had detectable hormone concentrations. The interstitial tissue is suggested as the source of pregnenolone.

INTRODUCTION

The breeding of the squirrel monkey (*Saimiri sciureus*) in captivity has met with only partial success. This fact may be related to the scarcity of objective data concerning the reproductive physiology of this New World primate (Beischer & Furry, 1964; Rosenblum, 1968; Hutchinson, 1970). Because of this, the majority of the animals used in research have been obtained from their natural habitat with all the problems that such supply creates.

Some attempts have been made to obtain ovulation and pregnancy after treatment with exogenous hormones (Bennett, 1967a, b). Following the artificial insemination of three adult monkeys, fertilized eggs were recovered at the two-cell stage from the Fallopian tubes of two monkeys and at the two-cell and four-cell stage from the uterus in the third animal (Bennett, 1967b). As in other species, the administration of exogenous gonadotrophins may result in unphysiologically high steroid secretion. In the rat, following PMSG–HCG treatment, not only is the progesterone concentration elevated to levels found only in pregnant rats (Fajer, 1968) but oestrogen secretion may be abnormally high as deciduomata formation is inhibited (Guillet & Rennels, 1964). In man, artificial induction of ovulation very often results in over-stimulation with ovarian enlargement and cyst formation (Rosemberg, 1968).

This paper describes the pregnenolone (3 β -hydroxypregn-5-en-20-one) and progesterone (pregn-4-ene-3,20-dione) secretion by artificially stimulated ovaries

of immature animals as part of a larger project on the ovarian physiology in the squirrel monkey.

MATERIALS AND METHODS

Animals

Twenty immature female squirrel monkeys of Peruvian and Brazilian phenotype (Hill, 1960) with an average weight of 634 g were used. The animals were obtained from commercial suppliers and were acclimatized to laboratory conditions for at least 1 week before treatment. Purina monkey chow and water were always available and a vitamin supplement was administered orally, twice weekly.

Treatment

Ovulation was induced by the intramuscular administration twice daily for 9 days, of 50 i.u. of PMSG (Equinex, Ayerst Labs), supplemented during Days 6 to 9 by twice daily intramuscular injections of 250 i.u. of HCG (Follutein, E. R. Squibb). The animals were killed 2 or 5 days after the last injection. Three animals were injected with an isotonic saline solution and used as controls.

Ovarian venous blood collection

The animals were anaesthetized with 35 mg/kg body wt of sodium pentobarbital (Nembutal, Abbott) administered intramuscularly.

The left femoral vein was cannulated for saline or drug administration. Rectal temperatures were continuously monitored with an electronic thermometer and maintained between 37 and 39° C with the help of an electric heating pad.

After heparinization (heparin sodium, U.S.P. grade, Fisher Scientific C. 5000 i.u./kg body wt, intravenously), blood was collected separately from each ovary directly from the ovarian vein using an appropriate polyethylene cannula. The collection time varied from 20 to 30 min for each sample. Blood samples were collected in tubes packed in crushed ice and later frozen at -20° C. Ovarian blood flow varied between 0.05 and 0.12 ml/min/ovary 2 days after treatment and between 0.04 and 0.06 ml/min/ovary 5 days after treatment.

Before vein cannulation, the ovaries were examined for sites of ovulation. At the end of the experiment, they were removed, weighed to the nearest mg and fixed for histological preparation.

Extraction of steroids

The samples were thawed and diluted to 10 ml with distilled water. To each sample were added 0.25 ml of 20% aqueous solution of NaOH and 4000 d/min [1,2-³H]progesterone (NET-208—S.A. 33.5 Ci/mmol, lot 400-054; New England Corp).

The samples were extracted three times with a total of 10 vol. of ethyl ether. The total extract was washed with a 0.1-vol. of 1% sodium carbonate solution, then with a 0.1-vol. of 1% acetic acid and 0.1 vol. of distilled water until neutral. After the addition of 1 ml of ethanol, the ether was evaporated under

reduced pressure in a rotary evaporator. The residue was partitioned four times (v/v) between petroleum ether (b.p. 30 to 60° C) and methanol:water (7:3, v/v). The aqueous methanol was evaporated to dryness with additional ethanol.

Thin-layer chromatography

The residue was spotted on silica gel plates (E. Merck, TLC-silica gel F-254, 0.25 mm thick) pre-washed by ascending chromatography for 24 hr with chloroform-methanol, methanol and ethyl ether. The chromatographic systems used were: A—benzene:ether, 1:1 (v/v); B—benzene:ether, 3:1 (v/v).

After development in System A, areas corresponding to authentic progesterone and pregnenolone were extracted in small funnels with 2 ml of a methanol-ether mixture (1:1), and the solvents were evaporated under nitrogen. The progesterone and pregnenolone samples were acetylated with acetic anhydride-pyridine (1:2) for 18 hr in the dark. After the addition of methanol, the reagents were evaporated and the residue rechromatographed on silica gel plates in System B.

The areas corresponding to progesterone and pregnenolone acetate were eluted and dried. The recoveries varied between 40 and 55%.

Gas liquid chromatograph (GLC)

After the first and the second chromatograms, steroid identity was further ascertained by GLC in a Model 402 F & M gas chromatograph using a 4-foot column of 3.8% SE-30 on Diatoport-S and a 6-foot column of QF-1 on Gaschrom Q (Applied Science Lab.).

Retention times were compared to that of authentic pregnenolone, progesterone and pregnenolone acetate, where necessary. Cholestane was used as an internal standard.

Quantitative determination of pregnenolone and progesterone

For routine purposes, progesterone and pregnenolone were eluted simultaneously after the first TLC development in System A and quantified using GLC with an SE-30 column against an internal standard of 11 β -hydroxyprogesterone (Heap, Holzbauer & Newport, 1966; Fajer & Holzbauer, 1968).

Samples of peripheral blood, of equal volume, when submitted to the same procedures showed no detectable peaks of pregnenolone or progesterone.

Results were adjusted to 100% recovery after determination of the radioactivity in an aliquot of the sample before GLC. Radioactivity was determined in toluene (50 mg/litre of POPOP and 4 mg/litre of PPO) using a Nuclear Chicago Liquid Scintillation counter model 720 with an efficiency of 30% for ^3H .

RESULTS

There was a significant increase in the ovarian and uterine weights in sixteen of the seventeen animals treated with PMSG and HCG (Tables 1 and 2). All organ weights fell within the same range regardless of the day of observation. In the

TABLE 1
ORGAN WEIGHTS OF ANIMALS 2 DAYS AFTER TREATMENT

Animal	Animal wt (g)	Uterus wt (mg)	Left ovary (mg)	Right ovary (mg)	Uterus (mg/kg body wt)	Ovary (mg/kg body wt)	
						Left	Right
(a) Pregnenolone and progesterone detectable							
A	625	NA*	430	600	NA	688	960
G	680	2140	424	742	3147	624	1091
H	710	2960	820	1446	4169	1155	2037
P	730	1270	545	1040	1740	747	1425
Q	570	1040	1374	850	1825	2411	1491
Mean	663	1853	719	936	2720	1125	1401
S.E.†	29	438	178	146	581	335	188
(b) Pregnenolone and progesterone not detectable							
B	585	NA	140	240	NA	239	410
(c) Control animals (mean of data from three monkeys)							
Mean	710	620	120	110	856	169	155
S.E.	70	124	9	17	95	7	17
<i>t</i> test, ‡ Group (a) versus Group (c)					<i>P</i> = 0.05	<i>P</i> < 0.02	<i>P</i> < 0.002

* NA—Not available.

† S.E.—Standard error of the mean.

‡ Values corrected for unequal variances (Bailey, 1959).

TABLE 2
ORGAN WEIGHTS* OF ANIMALS 5 DAYS AFTER TREATMENT

Animal	Animal wt (g)	Uterus wt (mg)	Left ovary (mg)	Right ovary (mg)	Uterus (mg/kg body wt)	Ovary (mg/kg body wt)	
						Left	Right
(a) Pregnenolone and progesterone detectable							
C	620	3350	1140	1050	5404	2323	1694
K	680	1960	1026	714	2882	1509	1050
L	780	1680	1216	896	2154	1559	1149
M	700	2120	712	1544	3029	1017	2206
O	610	1770	745	404	2902	1221	662
Mean	678	2176	968	922	3274	1526	1352
S.E.†	31	303	102	189	554	222	270
(b) Pregnenolone and progesterone not detectable							
D	630	1000	882	1030	1587	1400	1635
I	800	2650	102	274	3313	128	343
J	660	1780	1060	1080	2697	1606	1636
N	650	2470	674	610	3800	1037	938
R	590	500	359	327	847	608	554
S	760	1910	359	759	2513	839	999
Mean	682	1719	619	680	2460	936	1018
S.E.	33	339	148	341	445	219	219
<i>t</i> test‡ Group (a + b) versus Group (c) of Table 1 (untreated)					<i>P</i> < 0.001	<i>P</i> < 0.001	<i>P</i> < 0.001

* Controls in Table 1, Sub-group (c).

† S.E.—Standard error of the mean.

‡ Corrected for unequal variances. Although the means of Sub-group (a) and (b) appear to be different, the equality cannot be statistically rejected.

5-day group, no difference was seen between the organ weights of animals in which steroids were detected and those in which they were not detected (Table 2).

Only two out of six animals observed on Day 2 showed macroscopic signs of ovulation, while six out of eleven animals observed on Day 5 had ovulation sites. However, on histological examination, with one exception (Animal B, Table 1), all animals had ovulated and luteinization could be observed (Pl. 1, Figs. 1 and 2). The negative animal had a distinctly smaller ovary than any of the other animals of the same group.

Pregnenolone and progesterone were easily detectable in the ovarian

TABLE 3
PREGNENOLONE AND PROGESTERONE SECRETION 2 DAYS AFTER TREATMENT

Animal	Pregnenolone (μg)				Progesterone (μg)			
	Left ovary		Right ovary		Left ovary		Right ovary	
	Per ml	Per hr	Per ml	Per hr	Per ml	Per hr	Per ml	Per hr
A	0.35	1.70	0.14	1.10	0.73	3.40	0.41	3.30
G	0.16	0.57	NA*	NA	1.06	4.00	NA	NA
H	0.30	1.60	NA	NA	1.17	6.20	NA	NA
P	0.21	1.50	0.14	0.74	0.31	2.20	0.06	0.36
Q	0.48	4.60	0.56	4.49	0.59	6.10	0.15	1.20
Mean	0.31	1.99	0.28	2.11	0.77	4.38	0.21	1.62
S.E.†	0.06	0.68	0.14	1.19	0.16	0.78	0.11	0.87

* NA—Not available.

† S.E.—Standard error of the mean.

TABLE 4
PREGNENOLONE AND PROGESTERONE SECRETION 5 DAYS AFTER TREATMENT

Animal	Pregnenolone (μg)				Progesterone (μg)			
	Left ovary		Right ovary		Left ovary		Right ovary	
	Per ml	Per hr	Per ml	Per hr	Per ml	Per hr	Per ml	Per hr
C	1.56	7.80	2.57	9.80	4.56	22.80	4.78	22.00
K	0.08	0.38	0.11	0.30	0.10	0.47	0.21	0.60
L	0.64	1.83	0.08	0.25	0.42	1.25	0.09	0.29
M	0.14	0.71	0.03	0.11	0.11	0.53	0.03	0.11
O	1.44	4.60	0.00	0.00	0.84	2.70	0.00	0.00
Mean	0.77	3.08	0.56	2.09	1.90	5.55	1.02	4.60
S.E.*	0.31	1.39	0.50	1.93	0.85	4.33	0.95	4.35

* S.E.—Standard error of the mean.

venous blood of animals cannulated on Day 2. The average progesterone secretion was 0.77 $\mu\text{g}/\text{ml}$ of ovarian blood, or 4.38 $\mu\text{g}/\text{hr}/\text{left ovary}$. The range of these values was wide: 0.31 to 1.17 $\mu\text{g}/\text{ml}$, and 2.2 to 6.2 $\mu\text{g}/\text{hr}/\text{left ovary}$. Pregnenolone was present in lower concentrations with an average of 0.30 $\mu\text{g}/\text{ml}$ (range 0.16 to 0.48) and 1.99 $\mu\text{g}/\text{hr}/\text{left ovary}$ (range 0.57 to 4.60, Table 3).

Five days after the termination of treatment, only five out of eleven animals that had ovulated had detectable progesterone or pregnenolone in their ovarian blood (Table 4). Animal C (Table 4) had the highest values found in

this series of experiments, the ovary having a significantly higher degree of luteinization. Otherwise no histological differences could be observed between 'secretors' and 'non-secretors'. In general, the concentrations of progesterone found in this 5-day group tended to be lower than those of the previous group (Day 2). Excluding Animal C, the range of progesterone secretion was 0.10 to 0.84 $\mu\text{g/ml}$ or 0.47 to 2.7 $\mu\text{g/hr/left ovary}$. The values for pregnenolone also show a similar decline in concentration and degree of variability (Table 4).

DISCUSSION

The results of this study may assist in understanding the difficulties encountered in obtaining successful pregnancy in animals treated with PMSG and HCG (Bennett, 1967b). The treatment used in our study was effective in inducing ovulation in sixteen out of seventeen treated animals, confirming the earlier observations on a smaller group of animals by Bennett (1967a). However, only in eight animals could ovulation have been diagnosed by macroscopic examination of the ovary. Ovulation may be spread over a relatively long period of time (Dukelow, 1970), and the macroscopic signs of ovulation may disappear more rapidly in some animals than in others. Another possibility is that the extensive development of the follicles may not permit one to observe the developing corpora lutea by simple examination of the ovarian surface. Histological examination is then necessary for confirmation of ovulation and luteinization.

The treatment used in this study induced a marked growth of the ovaries (Tables 1 and 2). This growth was mainly due to extensive follicular development. The growth of the uterus would indicate that during the treatment, oestrogens were secreted as suggested by Bennett (1967b).

Extensive luteinization was evident in all animals, the antrum still being present in most of the developing corpora lutea, due to the large dimensions of the collapsed follicles. These luteinized ovaries were found to secrete progesterone and pregnenolone. Progesterone is the characteristic steroid of the corpus luteum; but pregnenolone, in the amounts found, is not a common steroid of ovarian venous blood (Dorfman & Ungar, 1965). However, pregnenolone has recently been described in rat ovarian venous blood during the oestrous cycle (Fajer & Holzbauer, 1968), and in hamster ovarian blood during pregnancy and lactation (Fajer, 1970). In the hamster, the suggested origin of pregnenolone is the ovarian interstitial tissue as there are no antral follicles or corpora lutea during lactation. At present, there are no data to extend this hypothesis to the squirrel monkey; however, New World monkeys (Dempsey, 1939) have a characteristically conspicuous interstitial gland (Pl. 1, Figs. 3 and 4).

The wide range of the values found for progesterone and pregnenolone suggests that ovarian secretion falls rapidly after the cessation of gonadotrophin treatment. This is supported by the finding that all five animals that ovulated had detectable steroids on Day 2 but that only five out of eleven had detectable steroids on Day 5 after ovulation. This being the case, progesterone is not present to balance the high oestrogen secretion and implantation and pregnancy cannot occur.

PLATE I

Haematoxylin and eosin stained sections of ovaries of *Saimiri sciureus*.

FIG. 1. Animal P 2 days after treatment with pmc-ncg. Extensive luteinization (LT). $\times 84$.

FIG. 2. Animal S 5 days after treatment. Formation of corpus luteum not completed (LT). $\times 84$.

FIG. 3. Non-treated animal. Characteristic interstitial tissue (IC) between granulosa (G) and theca (T) of a follicle and the primordial follicles (PF). $\times 240$.

FIG. 4. Animal C 5 days after treatment. Hypertrophied interstitial cells (IC) distinguishable from corpus luteum cells (LT). $\times 240$.

Further studies are now in progress to establish levels of exogenous gonadotrophins which are capable of producing a more balanced ovarian growth and hormonal production capable of supporting pregnancy.

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