CHAPTER IV

SITE OF ACTION OF PROGESTATIONAL STEROIDS
INTRODUCTION

The progestational steroids are highly effective in the control of conception. A number of mechanisms for their action at different sites in the body have been put forth (see page 9-25 of this thesis). Of these numerous mechanisms, the suppression of pituitary gonadotropins and inhibition of ovulation was proposed by Pincus (1957) as the principal mechanism of action of the progestagen-estrogen combinations. Over the years, the dose of the progestagens and the estrogens in the contraceptive preparations have been reduced to minimize the side-effects associated with their use. Low doses of progestagens alone are also being investigated for the control of fertility. The low dose progestagens have been suggested to act without inhibition of ovulation (Martinez-Manautou et al. 1966), while in some cases these act by inhibition of ovulation (Taymor et al. 1971). It thus becomes evident that the site of action of a progestational steroid is greatly dependent upon the dosage of the drug used and the mode of administration. In order to elucidate the site of action
of different doses of the progestational steroids, there is a need for an experimental animal model. Monkey - a non-human primate - has endocrinological and nervous behaviour very similar to the human. It thus offered a suitable model for the study of mode of action of progestational steroids. The inhibition of ovulation and corpus luteum function could be evaluated by the measurement of plasma progesterone levels. The work presented in this chapter deals with the development of a sensitive assay for the estimation of plasma progesterone and the assay of progesterone levels in monkeys after the administration of Enavid. The work also provided guidelines for the study of the mode of action of progestational steroids in an animal model.

**MATERIALS AND METHODS**

**Animals and sample collection:** Sexually mature female rhesus monkeys (Macaca mulatta) were chosen on the basis of their regular menstrual cycles varying from 25 - 28 days. They were healthy and weighed between 9-10 kg. Their menstrual cycles were followed at least for 3 successive cycles.
before they were used for the study. Monkeys were
individually caged in airconditioned quarters and
were fed on pellet diet supplied by Hindustan Lever
Ltd., Bombay. This diet was supplemented by fresh
fruits and vegetables. Water was provided ad libitum.
Blood samples were collected by femoral puncture
either daily or on alternate days, using heparinized
syringes, in the morning before the animals were
fed. The blood samples were immediately centrifuged
and the plasma was separated and stored frozen at
\(-10^\circ\text{C}\) until assayed for progesterone. To minimize
reduction in haematocrit values 100 mg of iron-
dextran (Imferon) was given intramuscularly once
every week throughout the experimental cycles to all
the animals. The first blood sample for each menstrual
cycle was drawn on day 3 of the cycle and the last
a day before the onset of next menstrual bleeding.

Treatment of the monkeys: The control monkeys did not
receive any drug while the treated monkeys were
administered "Enavid" tablets each containing 2.5 mg
norethynodrel and 0.1 mg mestranol daily. The
tablet was dissolved in 1.0 ml of olive oil and
administered orally to each monkey through a stomach
tube. The treatment was given to monkeys for two
successive cycles but the effect of the drug on
progesterone levels was studied in the 2nd
treatment cycle only.

**Competitive protein binding assay for progesterone** -
The principle underlying the competitive protein
binding assay is the competition between the hot and
the cold steroid for the binding sites on the plasma
protein which has high specificity and affinity for
the steroid. Competitive protein binding assay of
progesterone is based on the above principle and isexplained as follows. Progesterone, like cortisol
and some other steroids, seems to be specifically bound
though in subvalent levels to an $\alpha_1$-globulin
fraction of serum proteins. This protein has been
called "Transcortin" (Slanwhite and Sandberg, 1959)
or "corticosteroid binding globulin" (CBG) (Daughaday,
1958). It is present in the plasma of almost all
mammalian species; its level being very high during
pregnancy or during estrogen treatment. For the
assay of progesterone, the plasma is diluted and
the corticosterone binding globulin is saturated with
$^3$H-corticosterone. The binding sites on the cortico-
steroid binding globulin saturated with $^3$H-cortico-
sterone (CBG-$^3$HB) can be competed for by non-labelled
progesterone. The procedure is outlined as follows
in three steps.

**Step I.** $\text{CBG} + ^3\text{HB} + P \rightleftharpoons \text{CBGP} + ^3\text{HB}$
This step shows that after addition of cold progesterone (P) to diluted CBG solution saturated with $^3$H-corticosterone an equilibrium is set up between bound $^3$H-corticosterone ($^3$HB) and bound progesterone.

Step II. Adsorption of free progesterone and $^3$H-corticosterone by adding florisil.

Step III. The separation of $^3$H-corticosterone bound to CBG and counting.

If the amount of $^3$H-corticosterone bound to CBG with which the process started is known, and if known amounts of progesterone are used to compete for the binding sites, a standard curve can be established. When the unknown amount of progesterone extracted from the plasma samples is allowed to compete with a known amount of tritiated corticosterone, the amount of progesterone present can be calculated with reference to the standard curve.

Source of CBG: The source of CBG used was male dog plasma in 1.0% concentration, human pregnancy plasma in 1.0, 0.2, 0.15, 0.1 and 0.075% concentrations and monkey plasma from non-pregnant monkey in 0.1% concentration. Monkeys were also treated with "Enavid" an oral contraceptive containing 2.5 mg
norethynodrel and 0.1 mg mestranol for 3 months. Prior to the venipuncture the monkeys were given dexamethasone for adrenal suppression. Plasma collected from such treated monkeys was used to run a standard curve in concentrations from 1.0, 0.2, 0.15, 0.1 and 0.075.

One venipuncture blood was drawn into heparinized syringes and immediately centrifuged to separate plasma. Appropriate aliquots of plasma were transferred into sample tubes, frozen and stored at -10°C and used as a source of CBG.

**Radioactive steroids**— 1,2-^3^H-corticosterone (Sp. Act. 44 Ci/mM) and 1,2-^3^H-progesterone (Sp. Ac. 50.0 Ci/mM) were obtained from New England Nuclear, Boston, Mass.

**Non-Radioactive Steroids**— Non-radioactive progesterone was obtained from Sigma Chemical Company and was purified by repeated crystallization using methanol and water as solvent pair. The crystalline progesterone was dried overnight in a vacuum desiccator and used at different concentrations in the standard curve.

**Counting procedures**— The counting medium used was prepared by dissolving 100.0 ml of Biosolve and 42.0 ml of Liquifluor (Beckman Instrument Co.) in
l litre of redistilled toluene. The samples were counted in a Packard Scintillation Counter Model No. 3314 which gave counting efficiency of 28 - 30 \%.

The composition of diethyl counting medium is given in Chapter I.

**Assay procedure**— The progesterone estimation was carried out by competitive protein binding assay according to the procedure given by Neill, Johansson, Datta and Knobil (1967).

The CBG-corticosterone 1,2-^H (CBG-^H) was prepared as follows: 4 \mu l of \(^{3}\text{H}\)-corticosterone in ethanol were transferred to a reagent bottle of 150 ml capacity and evaporated under a gentle stream of nitrogen. 100 ml of distilled water was added to the bottle, shaken well and placed in water bath at 45°C for 5 min. to bring the \(^{3}\text{H}\)-corticosterone into solution. 1.0 ml of male dog plasma was added and the solution was refrigerated for 30 min before use.

Non-radioactive progesterone, crystallized and dried was used for making standard solution of progesterone in different concentrations of 0.2, 1, 2, 3, 4, 5, 10 and 50 ng/0.06 ml of absolute alcohol. The standards were stored in a refrigerator at 4°C.

**Standard Curve**— 50 \mu l aliquots of progesterone solution in absolute ethanol containing 0, 0.2, 1, 2,
3, 4, 5, 10 and 50 ng of progesterone respectively were added in duplicate to 12 x 100 mm disposable culture tubes and evaporated to dryness. 1.0 ml of CBG-\(^3\)H solution was added to the tubes which were shaken on Vortex mixer for 15 sec. and placed in a water bath at 45\(^0\)C for 5 min to bring the progesterone into solution. They were then mixed on vortex mixer for 15 sec and transferred to an ice bath for 30 min to increase the affinity of CBG for steroids (Murphy, Engelberg and Pattee 1963).

The unbound labelled corticosterone was removed from CBG solution by adsorption to Florisil. Florisil (40 mg) was added to each tube which was then immediately mixed on vortex mixer for exactly 30 sec and returned to the ice bath. When mixing of all the tubes was completed, 0.5 ml of supernatant from each tube was added to 15.0 ml scintillation medium in a vial and counted. A standard curve for % bound corticosterone against different concentrations of progesterone was plotted.

**Radioimmunoassay for progesterone**— Radioimmunoassay is a highly specific and sensitive method for the measurement of picogram quantities of steroids in plasma. The principle of radioimmunoassay is the same as that of competitive protein binding assay with the difference that in radioimmunoassay the binding protein
is antiserum containing specific antibodies raised against a steroid. This antiserum is raised against the steroid (antigen) by conjugating it to a protein, emulsifying it with complete Freund's adjuvant and injecting subcutaneously into the host animal (rabbit, sheep, etc.). The antiserum is obtained by bleeding the host animal after it has developed a high titer antiserum.

**Antiserum:** The antiserum was supplied by Dr. K. Sundaram, Population Council, Biomedical Division, New York. It was raised against \(11\alpha\)-hydroxyprogesterone linked to bovine serum albumin and is highly specific for progesterone (Midgley and Niswender 1970). The dilution of antiserum used was 1 : 5000 and at this dilution it bound 50 - 60% of the \(^3\)H-progesterone (1 \(\mu\)g/10 ml buffer).

**Buffer** - 0.1 M phosphate buffer (pH 7.2) containing 0.9% NaCl and 0.1% Knox unflavoured gelatin was used throughout the assay.

**Dextran coated charcoal solution** - Norit A (250 mg) and Dextran T-70 (25 mg) were suspended in 50 ml of gelatin-phosphate buffer. Fine particles were removed from the charcoal by repeated washing with double distilled water and drying at 100°C for 2 - 3 hours.

**Solvents** - All the solvents that were used in the assay
were of high analytical grade. Anhydrous diethyl ether and double glass distilled water were used.

Glassware - Disposable assay tubes were used. The other glassware that was used was dipped in chromic acid overnight and then washed with distilled water repeatedly to get rid of all traces of acid. Before using for assay the glassware was rinsed twice with double distilled water and methanol and dried.

Radioactive progesterone - Specifically labelled progesterone in 1, 2, 6, 7-position was obtained from New England Nuclear, Boston, Mass. It had a high specific activity of 100 Ci/mM. 10 μc of 1, 2, 6, 7-^3H-progesterone was dissolved in 100 ml gelatin-phosphate buffer to make a solution of ^3H-progesterone. This is referred to as 'Hot' buffer.

Standard Curve - Progesterone in concentration of 0, 25, 50, 100, 200, 400 and 800 pg/50 μl ethanol was transferred in duplicate to disposable 12 x 75 mm culture tubes. 3.0 ml of anhydrous diethyl ether was added to each tube, and the solvent evaporated by placing the tubes in a water bath at 45°C. Antiserum 0.15 ml in gelatin phosphate buffer at the dilution of 1 : 5,000 was added to each tube and mixed on Vortex mixer for 10 sec to bring the progesterone into solution. This was followed by
adding 0.15 ml of 'Rot' buffer to each tube and mixing on Vortex mixer for 15 sec. The tubes were then left overnight in the refrigerator at 4°C for incubation. During incubation period, the $^3$H-progesterone and the non-radioactive progesterone compete for the binding sites on the antibodies. The greater the amount of cold progesterone present in the tubes the lower is the $^3$H-progesterone bound to antiserum.

Separation of free and bound steroids: The free and bound $^3$H-progesterone were separated by adding precooled 0.5 ml of dextran-coated charcoal solution to each tube. The tubes were mixed on a Vortex mixer for 10 sec each and centrifuged in a refrigerated, centrifuge International model PR-2 at 3000 RPM for 10 min. The $^3$H-progesterone was adsorbed by dextran coated charcoal which settled at the bottom while the one bound to antiserum stayed in the supernatant. The supernatant was gently decanted into a vial. 10 ml of scintillation liquid (page 133) was added to each vial and was counted in a Tricarb Liquid Scintillation Spectrometer at a counting efficiency of 28 - 30%.

A standard curve was plotted on a semi-logarithmic paper with progesterone concentrations
on the logarithmic scale on abscissa and the % of the counts bound at 0 progesterone mass on the ordinate.

**Sample extraction and assay:** Plasma samples 0.2 ml from follicular phase and 0.1 ml from luteal phase of unknown progesterone concentration were added in duplicate to each 15 x 125 mm test tubes and diluted with double distilled water to a volume of 0.5 ml. Extraction was carried out using 5.0 ml of anhydrous analytical grade diethyl ether per sample and mixing it on Vortex mixer for 1 min. This was followed by centrifugation to ensure clear separation of the two phases. The ether extract was then transferred to 12 x 100 mm assay tubes by pasteur pipettes and evaporated in a water bath at 45°C. From here onwards the samples were processed as described for the standard curve, with one set of tubes for a standard curve in the beginning and one at the end. The progesterone value for each unknown plasma sample was determined by reading the value from the standard curve plotted by using the mean values of the two set of tubes. A standard curve using mean value of 5 sets of observations is given in Fig.1.

To keep a check on the assay system 0.5 ml of double distilled water was assayed along with
FIG. 1

The per cent bound $^3$H-progesterone is plotted against the log of progesterone mass.
PERCENT OF COUNTS BOUND AT 0 MASS (MEAN ± SD)
the unknown plasma sample. A value higher than zero of the standard curve obtained with water blank necessitates the repeating of the assay.

**Recovery correction:** Procedural loss was corrected by adding 500 cpm of 1,2-^H-progesterone in ethanol to 2 extraction tubes. The solvent was evaporated and 0.2 ml male plasma was added to each tube and extracted as per sample plasma. The extract was transferred to a vial, evaporated and counted. The % recovery obtained was usually 95 - 98%.

**Reproducibility and precision of the assay:** The day-to-day reproducibility and precision of the assay was checked as follows: 200 pg and 800 pg of progesterone were dried in duplicate and 0.2 ml of male or ovariectomized female plasma was added to them. The samples were assayed together with unknown plasma samples. The blank in each case was male or ovariectomized plasma without any added progesterone. The value obtained was read from the standard curve run with each assay. The recovery with 200 pg and 800 pg was 0.2 ± 0.08 SD and 0.8 ± 0.07 SD (n = 6).
RESULTS

Use of male dog serum for CPB assay:— The corticosteroid binding globulin or transcortin, present in the male dog plasma has been used as a binding protein in the competitive binding assay for progesterone (Neill, Johansson, Datta and Knobil, 1967). It was proposed to measure plasma progesterone levels of non-pregnant monkeys in both follicular and luteal phase of the cycle. The assay should measure as low as 2 ng/ml plasma. The standard curve 1 - 4 ng progesterone using 1 ml male dog plasma/100 ml water as the binding protein gave a curve which is shown in Fig. 2. It may be seen that the curve obtained did not give satisfactory displacement at 1 - 4 ng progesterone amounts. However, significant drop could be obtained when the progesterone concentrations used were 10 to 50 ng as given in Fig. 3. Due to the low sensitivity of the assay using male dog serum this binding protein could not be used for the assay.

Use of pregnancy plasma for CPB assay:— Since the levels of corticosteroid binding globulin are known to rise during pregnancy, the pregnancy plasma was used as the binding protein for competitive protein
FIG. 2

The % bound $^3$H-corticosterone is plotted against the progesterone concentrations of 1 - 5 ng. The source of CBG is 1% male dog plasma.
FIG. 3

The % bound \(^3\)H-corticosterone is plotted against the progesterone concentration of 5 - 50 ng. The source of CBG is 14% male dog plasma.
binding assay. With the use of 1% pregnancy plasma, in the standard curve ranging from 1 - 50 ng, (Fig. 4), it was seen that the standard curve remained flat from 1 - 5 ng progesterone concentration, while from 5 - 50 it showed a significant drop in per cent bound corticosterone. Fig. 5 shows the pattern of standard curves obtained using decreasing amounts of pregnancy plasma 0.2, 0.15, 0.1 and 0.075% in distilled water. The use of low amounts of pregnancy plasma gave a displacement of the bound steroid to the extent of 50 to 60% from 1 - 4 ng progesterone. The use of 0.075% of pregnancy plasma gave a standard curve which was more linear and gave a higher displacement from 1 to 4 ng. However, the required 50 - 60% displacement at 2 ng level (Stone, Nakamura, Mishell and Thorneycroft, 1971) could not be obtained with 0.075% pregnancy plasma.

Use of monkey plasma as a source of CBG for CFB assay: Monkey was treated with Enavid for 6 months and before venipuncture it was administered dexamethasone (3.0 mg) in a dose of 0.5 mg every six hours. Different amounts of plasma from the monkey ranging from 1.0, 0.5, 0.4, 0.3, 0.2, 0.1 and 0.075% were used for standard curve (Fig. 6). The use of 0.075% plasma for standard curve gave 30%
FIG. 4

The % bound $^3$H-corticosterone is plotted against the progesterone concentrations of 0.2 - 50 ng. The source of CBG is 1% of human pregnancy plasma.
**FIG. 5**

The % bound $^3$H-corticosterone is plotted against progesterone concentrations of 1 - 4 ng. The source of CSB is 0.2, 0.15, 0.1 and 0.075% of human pregnancy plasma.
FIG. 6

The % bound $^3$H-corticosterone is plotted against progesterone concentrations of 1 - 4 ng. The source of CBG is 1.0, 0.5, 0.4, 0.3, 0.2, 0.1 and 0.075% plasma of Enavid treated monkeys.
displacement of bound corticosterone at 4.0 ng with a uniform drop at 1, 2 and 3 ng. Plasma amount higher than 0.075 to 1.0% gave lesser displacement as the amount of plasma was increased. With the use of 1.0% plasma, the standard curve showed only 10% displacement of the bound corticosterone at 4 ng.

The standard curve obtained with the 0.1% plasma from an untreated monkey (Fig. 7) did not differ from one where 0.1% plasma from Enavid treated monkey was used.

Patterns of plasma progesterone levels in the normal menstrual cycle of monkeys: The average values for plasma progesterone levels as determined by the radioimmunoassay method in 5 normally cycling monkeys is presented in Fig. 8. The plasma progesterone values have been plotted against days before the onset of menstruation. It may be seen that during the proliferative phase of the cycle the plasma progesterone values were rather low. These were always less than 0.5 ng/ml plasma. At times there were slight fluctuations which increased the levels to about 0.5 ng/ml. The plasma progesterone levels began to rise from day 18 before the onset of next menstruation with some fluctuations. From day 16 before the onset of the next menstruation,
FIG. 7

The % bound $^3$H-corticosterone is plotted against progesterone concentration of 1 - 4 ng. The source of CBG is 0.1% of untreated monkey plasma.
the levels rose sharply and on day 14 before the beginning of next menstruation the values reached 3.2 ng/ml. From thereon it continued to maintain a similar or somewhat higher level of progesterone till 6 days before the next menstruation. The peak of progesterone concentration reached a value of about 4.1 ng/ml plasma on day 10 before the onset of next menstruation. The progesterone level declined abruptly on day 5 before the next menstruation reaching a low value of 0.4 ng/ml plasma. From this time onwards till the beginning of the next menstruation, values continued to remain low.

**Plasma progesterone levels during the menstrual cycle of individual monkeys:** The mean plasma progesterone levels during the menstrual cycle of 5 monkeys are presented in Fig. 8. Other normal patterns of plasma progesterone during the menstrual cycle may also be seen in Fig. 10, 11 and 12. These normal patterns in general show very low levels of progesterone in the proliferative phase, generally less than 0.3 ng/ml, with considerable fluctuation. The levels then rise from about day 8-11 onwards, remain elevated with some fluctuations till about day 22 - 25 followed by a decline. Considerable day-to-day fluctuations in the daily plasma progesterone levels have been noted. Fig. 9A and 9B show plasma progesterone levels during four individual cycles with
FIG. 8

Mean peripheral plasma progesterone concentrations of five cycles plotted against days before the onset of menstruation. Vertical lines indicate Mean ± S.D.
extreme variations compared with the normal pattern described above.

The plasma progesterone levels in case of monkey M-8 (Fig. 9A) showed a rise to a level of 2.4 ng/ml starting from day 7 and continued to rise upto day 13. From day 13 - 19, it maintained a high level with fluctuations. From day 19 onwards it gradually declined to a low level of 0.3 ng/ml on day 25.

M-48 (Fig. 9A): The plasma progesterone levels in the case of M-48 showed two distinct sharp peaks – one on day 9 and the other on day 14. The plasma progesterone levels were below 0.2 ng/ml upto day 7. On day 8 these abruptly increased and reached a sharp peak on day 9 followed by a sharp decline on day 10 to about 0.6 ng/ml. From day 13 these again increased and reached a low peak of 1.9 ng/ml, followed by a slow gradual decline upto day 21.

M-49 (Fig. 9B): During the follicular phase upto day 7, the plasma progesterone values remained low, at or a little above 0.2 ng/ml plasma. From day 7 these continued to rise and reached a peak of 3.3 ng/ml. From there onwards the progesterone levels remained high with fluctuations upto day 16, except that there was a sharp dip on day 12, to a
Pattern of plasma progesterone levels in the individual normally menstruating monkeys M-8 and M-43, with extreme fluctuations in the progesterone concentration during the cycle.
M. 8 CONTROL CYCLE
m. MENSTRUAL BLEEDING

DAY OF CYCLE

M. 48 CONTROL CYCLE
m. MENSTRUAL BLEEDING

DILY OF CYCLE
level of 0.2 ng/ml. From day 16, it abruptly declined to about 0.9 ng/ml. This level was maintained up to day 23, and then became low of the order of 0.3 ng/ml on day 24.

M-1. The follicular phase plasma progesterone levels in M-1 (Fig. 9B) fluctuated between 0.3 and 0.6 ng/ml up to day 12 with the exception of day 3 when it was 1.1 ng/ml plasma. From day 12 progesterone levels rose up to 2.4 ng/ml with a further increase to 3.0 ng/ml on day 14. Between day 14 and day 23 progesterone levels remained high, reaching a peak of 4.7 ng/ml on day 17 and 5.4 ng/ml on day 21. These high progesterone levels were interrupted on day 18 and 20 when progesterone concentration fell to a level of 0.2 ng/ml. From day 23 the levels declined gradually up to day 28 to a low level of 0.2 ng/ml plasma.

Levels of plasma progesterone in the normal menstrual cycle and after the administration of Enavid: The patterns of plasma progesterone levels during a control cycle, 2nd treatment cycle and 2nd post-treatment cycle of monkey M-11 are presented in Fig. 10. The control cycle exhibited a normal pattern of progesterone levels as described
FIG. 9B

Pattern of plasma progesterone levels of individual normally menstruating monkeys M-49 and M-1 with extreme fluctuation in the progesterone concentration during the cycle.
earlier. The follicular phase progesterone levels were below 0.3 ng/ml till day 8 with a slight elevation on day 7. From day 9 progesterone levels continued to rise and reached a peak of 4.5 ng/ml on day 19. From then on the progesterone levels started declining gradually up to day 28, when these fell to a low level of 0.61 ng/ml. Between day 15 and day 24, i.e., during the luteal phase, the plasma progesterone concentration remained high, of the order of about 3.2 - 4.5 ng/ml.

After the administration of Enavid from day 5 to 25 of the menstrual cycle, the plasma progesterone levels were measured in the 2nd month of treatment. The length of the cycle was reduced from 28 days to 25 days, and the progesterone levels during the treatment cycle were suppressed. During follicular phase of the cycle, the progesterone levels were low and showed a considerable fluctuation ranging from 0.1 ng/ml to 1.2 ng/ml. In the luteal phase of the cycle, the progesterone levels were exceptionally low ranging from 0.1 ng/ml to 0.21 ng/ml, with very little fluctuations. The predominant progesterone rise during the luteal phase as seen in the control cycle was abolished.

After the treatment with Enavid for 2 months,
Peripheral plasma progesterone concentrations in monkey M-9, in the normal menstrual cycle and in the 2nd treatment cycle with "Enavid".
the drug administration was stopped and the plasma progesterone levels in the 2nd post-treatment cycle were measured. The progesterone levels showed a pattern similar to that seen in the normal ovulatory cycle. The follicular phase levels of progesterone were 0.2 ng/ml or lower till day 8. From then on progesterone concentration continued to rise reaching a peak of 3.4 ng/ml on day 19, followed by a gradual decline up to day 26 to a level of 0.6 ng/ml which continued to persist as such up to day 28.

Fig. 11 shows the pattern of plasma progesterone concentration in a normal ovulatory cycle and in the 2nd treatment cycle in monkey M-10. The plasma progesterone concentration during normal menstrual cycle of M-10 continued to rise steadily from day 2 and reached a peak of 3.7 ng/ml on day 15 followed by a gradual decline up to day 20 to a level of 0.6 ng/ml. The progesterone level continued to remain low from day 20 to day 25. The length of the cycle was 26 days.

In the 2nd treatment cycle Enavid was given from day 5 to 25 of the cycle and plasma progesterone levels were measured. It was seen that progesterone concentration did not rise in the 2nd half of the cycle, as seen in the control cycle. Throughout the treatment cycle the progesterone concentration remained
FIG. 11

Peripheral plasma progesterone concentrations in monkey M-10 in the normal menstrual cycle and in the 2nd cycle of treatment with "Enavid".
M-10. CONTROL CYCLE
m. MENSTRUAL BLEEDING

M-10. 2nd TREATMENT CYCLE
m. MENSTRUAL BLEEDING
low and varied between 0.8 ng/ml and about 0.4 ng/ml. The length of the cycle was reduced from 25 in the control cycle to 21 in the treatment cycle.

Fig. 12 shows the pattern of plasma progesterone in monkey M-9 during a control cycle and in the 2nd Enavid treated cycle. The progesterone concentration in the follicular phase was below 0.3 ng/ml up to day 8 of the cycle. From thereon the progesterone concentration increased abruptly to 3.3 ng/ml on day 11. From day 11 to day 20, progesterone maintained a high level of 3.3 - 4.00 ng/ml. The progesterone level declined abruptly from day 20 to a very low level of 0.3 ng/ml. Thereafter low levels were maintained till day 27. When Enavid was administered from day 5 to day 25, the plasma progesterone levels were suppressed throughout the cycle. The plasma concentration varied between 0.6 and 0.3 ng/ml plasma throughout the whole cycle.
FIG. 12

Peripheral plasma progesterone concentrations in monkey, M-9, in the normal menstrual cycle and 2nd treatment cycle with "Enavid".
M-9. CONTROL CYCLE
m. MENSTRUAL BLEEDING

M-9. 2nd TREATMENT CYCLE
m. MENSTRUAL BLEEDING
DISCUSSION

Although it is known that progestagen-estrogen combination oral contraceptives act by suppression of pituitary gonadotropins and consequent inhibition of ovulation, yet the precise mechanisms involved in their action and the effect of change in the dosage of the contraceptives on their action are not fully understood. There is a need to develop animal models in which these mechanisms could be elucidated. To study the inhibition of ovulation and corpus luteum function, the earlier studies (Stevens and Vorys, 1967; Diczfalusy et al. 1969; and Gellar, 1969) investigated the urinary LH and pregnanediol. Pregnanediol is a metabolite of progesterone and a study of its levels in the urine does not reflect the true picture of the endogenous levels of progesterone. To assess the changes in progesterone secretion after the administration of oral contraceptives, a study of the levels of progesterone during the cycle provided an extremely useful parameter. The investigations were directed to the development of a sensitive assay for the measurement of plasma progesterone levels and its application to the study of progesterone levels after the administration of oral contraceptives.
Development of a sensitive and specific assay for progesterone

The principle of competitive protein binding of steroids (Murphy, Engelberg and Pattee, 1963) has provided a useful approach to the development of a sensitive competitive protein binding assay for progesterone (Murphy, 1967; Neill, Johansson, Datta and Knobloch, 1967). Investigations were carried out to test the suitability of male dog plasma as a source of binding protein for the progesterone assay. Significant displacement of bound corticosterone could be achieved only when progesterone concentrations of 10-50 ng were used, while at 1-4 ng the assay did not give any satisfactory displacement, thus limiting the sensitivity of the assay. In view of these considerations the use of male dog plasma for a sensitive plasma progesterone determination was abandoned.

Next, the use of pregnancy plasma as a source of binding protein for the assay of plasma progesterone was examined. Corticosterone binding globulin levels are known to be elevated (Slaunwhite and Sandberg, 1959) during pregnancy. Making use of this information and the easy availability of pregnancy plasma, it was used as a source of binding protein for the progesterone assay. Pregnancy plasma was used in different concentrations ranging from 0.075, 0.1, 0.2, 0.3, 0.4, 0.5 and
1.0 per cent for standard curve of the assay. When
1.0% pregnancy plasma was used, the standard curve
ranging from 0.25 - 50 ng remained flat in the
region of 0.25 - 5 ng indicating no displacement
of bound $^3$H-corticosterone. The displacement was
evident only at higher concentrations of progesterone 5 to 50 ng in the standard curve. When
pregnancy plasma was used in decreasing concentra-
tion 0.5% to 0.075% an increasing displacement of
bound $^3$H-corticosterone was observed but still at
the lowest concentration of 0.075% the displacement
was only of the order of 10 - 15% at 1 ng progesterone.
This poor displacement at the lowest concentration
(0.075%) of plasma or a flat curve from 0.25 ng -
5 ng when 1.0% plasma was used indicated the presence
of other binding proteins besides the corticosteroid
binding globulin. Rosenthal, Slanwhite and
Sandberg (1969) reported the presence of unknown
binding protein for progesterone during pregnancy
besides the elevated levels of corticosteroid binding
globulin. It is probably due to the presence of
this unknown progesterone binder, to which the low
amounts of progesterone used in the standard curve
bind, thus giving either no displacement at a low
dilution of 1.0% plasma or a very poor displacement.
at high dilution of 0.075% of plasma. In view of these considerations pregnancy plasma could not be used as a source of binding protein for the progesterone assay.

Corticosteroid binding globulins are known to increase during estrogen treatment and pregnancy (Sandberg and Slamwhite 1959). The most sensitive plasma available for progesterone binding assay can be obtained from patients treated with combined oral contraceptive and dexamethasone (Yoshimi and Lipsett, 1968). In view of the non-availability of plasma from such treated women the possibility of using plasma from oral contraceptive treated monkeys with dexamethasone given before the venipuncture was explored. The use of plasma from such monkeys in both high and low concentration from (0.075 - 1.0%) did not give proper displacement of about 50 - 60% at 2 ng level (Stone et al. 1971). As a result of it the use of monkey plasma was abandoned for the assay of progesterone.

At this stage of work, studies on the development of specific and highly sensitive radioimmunoassay for progesterone estimation were reported. (Abraham, Swerdloff, Tulochinksky and Odell, 1971; Furuyama and Nuegent, 1971). However, the assay lacked specifi-
city and involved a chromatographic separation of progesterone before its estimation. Thornycroft and Stone (1972) developed an antiserum against 11α-hydroxyprogesterone linked to bovine serum albumin (BSA) which was more specific than antisera produced against 20 or 3-keto linkage of progesterone to BSA (Midgley and Niswender, 1970). The high specificity of the antiserum raised against 11α-hydroxy progesterone linked to BSA has been attributed to the fact that conjugation of BSA at 11-position leaves both ends of the steroid free to confer specificity to the antiserum.

Two different lots of antisera - one from Dr. I.H. Thornycroft of Los Angeles and the other from Dr. K. Sundaram, New York, raised against 11α-hydroxy progesterone linked to BSA - were obtained and used for the development of a sensitive radio-immunoassay for the estimation of plasma progesterone. The antiserum obtained from Dr. Thornycroft gave 56% binding at a low dilution of 1:250 on arrival, while the dilution at which it was used by Dr. Thornycroft was 1:5000. The antiserum received from Dr. K. Sundaram bound 56% of progesterone
at 1:5000 dilution (Fig. 13). This antiserum was thus used in all subsequent studies. The sensitivity of the radioimmunoassay for progesterone thus developed was as low as 25 pg. The assay was highly specific and did not require the chromatographic separation of progesterone prior to assay. It gave accurate and reproducible results.

Patterns of plasma progesterone levels in monkeys during normal menstrual cycles and after the administration of Enovid.- The studies on the estimation of plasma progesterone in a number of normally menstruating monkeys showed that the patterns were qualitatively almost similar to those reported in women (Neill, Johansson, Datta and Knobil, 1967) except that considerable fluctuations in the day-to-day progesterone levels were observed. Quantitatively, the plasma progesterone levels of monkey and human are almost the same in the follicular phase of the cycle i.e. below 0.2 to 0.5 ng/ml, but in the luteal phase of the cycle the human progesterone levels are 3 - 4 times more than that seen in the monkey (Neill et al. 1967). Large fluctuations in the day-to-day progesterone levels during the menstrual cycle of normally menstruating...
FIG. 13

The per cent bound $^3$H-progesterone is plotted against the log of antisera dilutions. The antiseraum THR-R11-20 was supplied by Dr. I.H. Thorneycroft and the antiseraum S was supplied by Dr. K. Sundaram.
monkeys were observed by Neill et al. 1967a; Monroe, Atkinson and Knobil, 1970). Heap and Linzel (1966) have reported that plasma progesterone levels can be raised by laparotomy in goat suggesting that "stress" influences progesterone secretion in this species. The extreme fluctuations sometimes observed in plasma progesterone levels of monkeys during the cycle might possibly be due to stress of catching and restraining prior to blood sampling. Similar extreme variations in the plasma progesterone levels during the normal menstrual cycle of Bonnet monkey were reported by Stabenfeldt and Hendricks (1972). Significant decrease in plasma testosterone, LH and FSH levels during surgical operation in human males have been reported by Aono, Kurachi, Misutani, Hamanaka, Uozumi, Nakasima, Koshiyama and Matsumoto (1972).

A significant feature of the plasma progesterone levels in the normal menstrual cycle of the monkey was an early rise of progesterone from day 17 to 20 before the onset of menstruation. Johansson, Neill and Knobil (1968), while measuring periovulatory progesterone levels in rhesus monkeys, observed that increase in the progesterone level occurred at least 3 days before the day of ovulation and was signi-
Significantly high one day before ovulation. Precise correlation of plasma progesterone with LH (luteinizing hormone) and FSH (follicle stimulating hormone) and their role in the ovulation process need to be further elucidated. Significantly high progesterone levels in the luteal phase were observed 15 days before the onset of menstruation and continued to remain high in the range of 3.2 - 4.1 ng/ml for about 11 days, before these declined to low levels. While the pattern of plasma progesterone as described above was frequently encountered, in normally menstruating animals, there were other normally menstruating animals which showed progesterone patterns that were different from the normal ones (Fig. 9). They exhibited extreme fluctuation in plasma progesterone concentration during mid-luteal phase, which sometimes rose to one or more sharp peak or declined to as low a level as seen in the early follicular phase. The other variations seen in these cycles were a very early rise of progesterone in the follicular phase, or a very gradual decline in the luteal phase till the day of next menstruation. However, all these "extreme cycles" showed a definite rise of plasma progesterone in the luteal phase of the cycle,
which could be indicative of ovulation and subsequent corpus luteum formation.

The administration of "Enavid" containing 2.5 mg norethynodrel and 0.11 mg mestranol to monkeys from day 5 to day 25 of the menstrual cycle, suppressed the plasma progesterone levels in the luteal phase. Since the high progesterone levels of the luteal phase are indicative of ovulation and subsequent corpus luteum formation, it may be inferred that the administration of "Enavid" inhibited ovulation and prevented corpus luteum formation. The inhibitory effect of "Enavid" on ovulation could be due to the suppression of pituitary gonadotropins and/or due to its direct effect on the ovary. Ryan, Goss and Reid (1966) administered "Enovid" (5 mg norethynodrel + 75 μg mestranol) to women and found that the midcycle urinary FSH and LH peaks were abolished. Bell et al. (1967) administered "Enovid" (2.5 mg norethynodrel + 0.15 mg mestranol) and found a suppression of urinary FSH and LH levels. It thus appeared that norethynodrel in both 5.0 and 2.6 mg doses, was able to suppress both FSH and LH peaks. Mestranol alone in different doses of 50 - 100 μg did not produce any inhibition of LH peak (Vorys, Ullery and Stevens, 1965). These
observations taken together thus lead to the conclusion that the suppression of plasma progesterone by Enavid was a direct effect of the progestational steroids on the LH which inhibited ovulation and prevented corpus luteum formation. Besides this site of action of progestational steroids, evidence of their effect at the uterine level was put forth in Chapter 1 of this thesis. Concluding, one may say that the estimation of progesterone levels after the administration of progestational steroids, may be one of the very important parameters to study the effect of a drug on ovulation and that for such studies the monkey offers an attractive model.