Chapter 4

DISCUSSION

The fact that progesterone plays an important role on the process for the nidation of the fertilized ovum in the uterine mucosa has drawn vast attention of a number of investigators to the development of various techniques for the determination of this hormone. The production of endogeneous progesterone by the body has been assumed from the excretion of pregnanediol, the main metabolite in the urine (Klopper, 1957; Russel et al., 1960 and Kamolpatana, 1969). Although the determination of urinary pregnanediol reflects, at least to some extent, the secretion of progesterone by its sources and give clinically useful information, it is not the direct estimation of the parent hormone. In addition, collection of 24 hour urine specimen is known to cause inconvenience to the subjects to be studied. Much attention has been paid, therefore, to the direct determination of the hormone itself. Butt et al., (1951) was the first who performed the direct determination of progesterone in blood. Later, various techniques have been performed by a number of investigators i.e. spectrophotometric method (Zander & Simmer, 1954; Short, 1958 and Heap, 1964), gas-chromatographic method (Woolever & Goldfien, 1963; Wiest, 1967 and Riondel et al.,

1965), competitive protein binding method (Neill et al., 1967 and Johansson, 1969 b) and radioimmunoassay method (Abraham, 1971; Mikhail, 1972 and Saxena et al., 1974). The last mentioned method has been widely accepted in clinical and analytical laboratories in preference to many analytical methods of long standing mainly because they are frequently more practical and their reliability is usually high.

In an attempt to develop a specific antibody to progesterone, bovine serum albumin (BSA) was conjugated at the 3, 11 and 20 positions (Niswender & Midgley, 1970). It was shown that a fairly specific antibody to progesterone could be obtained by the conjugation of BSA at the 3 and 20 positions, but an even more specific antibody was obtained when the protein was conjugated at the 11 position.

It was anticipated that the conjugation of progesterone to BSA via the 11α-hydroxy position should provide a highly specific antibody (Niswender, 1970). This conjugation of the steroid molecule to the protein through a conformationally more neutral position presumably leaves both polar ends of the steroid and the angular methyl groups unobstructed and accessible for antibody complementation. The highly specific progesterone antibody can be used for an acceptable assay without chromatographic purification of the serum extraction (Mikhail, 1972).

Attempts have been made to standardize a radioimmunoassay of progesterone using the antibody provided by Mikhail (1972). It was found that (figure 7 , page 34) the antibody at a final concentration of 1:28,000 bound 50% of the labelled progesterone at the zero point and the usable range of the standard, using this dilution of antibody, was found to be between 0-500 pg of progesterone. This range could be applied for the measurement of serum progesterone needed in this study.

Various factors have to be carefully considered in order to optimised the condition to be used in any assay required. It has been often found, however, that the optimum conditions are not the most practical ones. Practicability was, therefore, gained the priority in this study.

The study on the effect of incubation temperature and time on the binary showed that at the end of 4 hours the suitable temperature of incubation which gave the initial binding of 40-60% was 4°C. This condition also gave the widest percentage drop of the value of the standard curve between 50 and 500 pg.

No significant increase in the binding was obtained when incubation time was increased to more than 4 hours at 4°C. However, for the practical advantage, overnight incubation (18-24 hours) was used in the present study. The increase of incubation temperature from 4°C to 20°C, 37°C and 50°C caused a decrease in the initial binding. Nevertheless the slope of the

curve was relatively steep for 20°C and 37°C temperature incubations and usable in comparison to the slope at 50°C which was found to be flatter and thus not useful for the present assay conditions.(table 1 , page 35).

Another important part needed in a radioimmunoassay is a suitable method for the separation of free and bound hormones. Various separation techniques are employed in radioimmunoassay of steroid hormones.

1. Differential migration of bound and free fractions. These methods included paper chromatoelectrophoresis (Berson, 1956), electrophoresis on starch gel, cellulose acetate or polyacrylamide (Hunter, 1964), wick chromatography (Orskov, 1967) and gel filtration (Haber, 1965).

2. Fractional precipitation of bound fraction. Different organic solvents were used by a number of investigators. There are ethanol-NaCl (Odell, 1965), ethanol (Heding, 1966), dioxan (Thomas, 1968) and polyethylene glycol (Desbuguois, 1971). Besides, salts i.e. sodium sulphate (Grodsky, 1960), ammonium sulphate (Chard, 1971) and acids i.e. trichloroacetic acid (Mitchell, 1967) were also used.

3. Double antibody methods. These techniques were widely used (Utiger, 1962; Morgan, 1963; Hales, 1963 and Daughaday, 1971) but the expense of obtaining the second antibody often prevent many workers from the application of the technique.

4. Solid-phase methods (Wide, 1966; Catt, 1967; Miles & Hales, 1968 and Donini, 1969).

5. Adsorption methods. These methods were commonly employed using many solid adsorbents such as coated charcoal (Herbert, 1965; Neill, 1967; Binoux & Odell, 1973 and Sand & Torjesen, 1973), silicates (Rosselin, 1966), anion-exchange resin (Meade, 1962 and Frenkel, 1966).

In this study the last listed technique was applied for the separation of free and bound hormones. Dextran-coated charcoal suspension is used as an adsorbent of the free steroid fraction. This separation method has many advantages, for examples, the nonspecific interference or blank values in an assay is low, it is fast as the time taken is usually less than 30 minutes; it is simple and requires no special skill.

It has been shown that the affinity of charcoal in adsorbing free steroids appears high for the least polar steroids (Binoux and Odell, 1973) and thus, progesterone will be well adsorbed. The dissociation of the bound hormone depends on the concentration of charcoal, the polarity of the hormone, the temperature and the time of incubation with the charcoal suspension.

The blank value normally does not exceed 2% of the total cpm added i.e. approximately 10,000 cpm of the tracer added, give blank value of 200 cpm. In the present study, 0.6% charcoal was the lowest possible concentration to give the acceptable blank value, the steepest curve as well as the high initial binding. Although, the higher the charcoal concentration, the more it would adsorb the free steroids, the excessive charcoal concentration is not well packed at the bottom of the tube and decrease the efficiency of the separation.

Other reliability criteria were studied. Figures 9 , 10 and 11 (pages 40 , 41 and 42) showed that none of the tested steroids interfrered with the binding of the standard progesterone and its antibody indicating no detectable cross-reaction. The accuracy of the method was found to be satisfactory only when the values of serum progesterone was 300 pg/ml or higher. Lower values were considered unreliable. The precision of the method, both for within and between-assay, was in an acceptable range since the coefficients of variation were less than 10% except that of pooled serum containing low values of progesterone which was considered unreliable as previously mentioned.

Although the limited amount of time available in this study did not allow a perfect standardization, the reliability of the method was acceptable and sufficiently high to be used in further studies.

Serum progesterone levels in normally menstruating women have been studied by various workers (Neill et al., 1967; Yoshimi & Lipsett, 1968; Wyman & Sommerveille, 1968; Johansson, 1969 a, b and Saxena et al., 1974). Progesterone levels remained low (<1 ng/ml) almost throughout the follicular phase but a marked increase was found following the estrogen peak, reaching a maximum at 5 to 8 days after the estrogen peak and fell rapidly towards the end of the luteal phase.

Interrelationship between blood LH, measured by radio-immunoassay, and serum progesterone at midcycle have been widely studied (Neill et al., 1967; Cargille et al., 1969 and Johansson & Wide, 1969). There is general agreement that the rise in serum progesterone levels does not commence until the LH levels begin to fall. Riondel et al., (1965) reported a mean progesterone concentration of 1.13 ng/ml during the follicular phase and 10.40 ng/ml during the luteal phase of the menstrual cycle. Van der Molen et al., (1965) using gas chromatography with flame ionization detection, reported progesterone concentrations of less than 1 ng/ml in pools of serum from days 1 to 10 of the menstrual cycle, 16.0 ng/ml from days 11 to 15, 12.50 ng/ml from

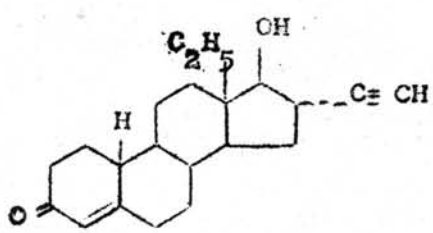
days 16 to 20 and 4.40 ng/ml from days 21 to the onset of menstruation. A more sensitive procedure of gas chromatography with electron capture detector (Van der Molen, 1965) yieled serum progesterone concentrations of 0.49 ng/ml (range : $<$ 0.15 - 1.10) on days 1 to 9; 1.30 ng/ml (range : $<$ 0.15 - 4.20) on days 7 to 16; 15.20 ng/ml (range : 7.90 - 20.60) on days 16 to 24 and 7.10 ng/ml (range : 1.00 - 17.90) on days 22 to 30 of the menstrual cycle. These last values reported agreed with those of Neill and Johansson (1967) who had found by the competitive protein binding method that the mean progesterone levels were 0.40 ng/ml with the ranges of 0.20 - 1.80 and 10.00 - 19.00 ng/ml in 3 to 5 days after the declination of serum LH concentration.

Radioimmunoassay methods for progesterone estimation in the menstrual cycle were used by a number of invetigators. Abraham (1971) showed that the serum progesterone levels in normally menstruating subjects was 0.54 ± 0.10 ng/ml ranging from 0.41-0.65 ng/ml during the follicular phase and 8.56 ± 4.66 ng/ml ranging from 1.48-13.50 ng/ml during the midluteal phase. Mikhail & de Villa (1972) reported that the serum progesterone levels during preovulatory period (days 2-10) was 0.50 ± 0.44 ng/ml with a range of 0.41-1.50 ng/ml and during the postovulatory period (days 19-23) was 12.10 ± 4.81 ng/ml ranging from 5.03-19.70 ng/ml.

The range of progesterone levels in the present study was 0.48-0.60 ng/ml during the follicular phase and that between days 16 to 29 of the cycle (luteal phase) progesterone level was found to be between 2.04-13.50 ng/ml.

Progesterone is used clinically in many ovarian and menstrual disorders, including functional uterine bleeding. Progesterone is often routinely given in the management of habitual and threatened abortion. Intensive research activity in the field of progesterone anologues has yielded not only a number of biological active stercids but also some interesting leads concerning the relationship between structure and hormonal activity.

Norgestrel (13β-ethyl 17α-ethynyl-17β-hydroxyon-4-ene-3-one) is a synthetic progestogen used as an oral contraceptive.



Norgestrel

Daily administration of small doses (50 to 75 µg) of Norgestrel have an antifertility effect (Foss et al., 1968). Wright et al., (1970) suggested that Norgestrel, while not interfering with the corpus luteum formation may perhaps inhibit the corpus luteum function, resulting in a decrease secretion of progesterone. When Norgestrel was continually given at a dose of 75 µg/day to women of proven fertility, it was found to be an efficient contraceptive with a failure rate of 2.1% to 3.6% (Eckstein, 1972). In another study (Kesseru, 1972), d -Norgestrel at 0.03 mg, the lowest dose of progestogon so far tested for contraception, was continuously administered to fertile women. The principal side effect was the frequent disturbance of the menstrual cycle pattern i.e. irregular cycle lengths and bleeding. The compound at this dose is thought to act primarily by interfering with sperm migration to the cervical mucus. Norgestrel apparently exerts its contraceptive action by several mechanisms which could be a reduction in the sperm penetrability of the cervical mucus and also an impairment of the corpus luteal function (Kesseru, 1972).

Martinez-Manautou and associates (1967) believed that the microdoses of progestogen do not inhibit ovulation or at least not so consistently as to account for the mode of contraception.

In the present study the effect of Norgestrel treatment on Thai women has been investigated. Ten Thai women who were of proven fertility taking Norgestrel as a contraceptive were studied and the blood samples were drawn for determinations of progesterone and estradiol.

It is generally agreed by various investigators (Abraham, 1971 and Saxena et al., 1974) that the levels of serum progesterone and estradiol during the midluteal phase of higher than 3 ng/ml and 150 pg/ml respectively indicates the occurance of ovulation and the formation of functioning corpus luteum. A finding of lower level of progesterone could possibly be resulted from

1. Anovulation One can expect no corpus luteum formation if there is no ovulation occuring in that cycle.

2. Persistent follicle In this case, the follicular phase of the cycle is longer than in normal cycles and one generally finds a high level of estradiol but a low level of progesterone.

3. Reduced steroid biosynthesis The level of progesterone can be lower if there is a block in the conversion of precursor steroids to progesterone.

The results from the present study suggested that the ovulation was inhibited in all of the Thai subjects studied except subject SG_9 (table 7, page 53) who presented her higher levels of both progesterone and estradiol than the above mentioned

levels in all three treated-cycles. Subject CB_9 also had this similar status but occurred only in one treated-cycle.

The levels of progesterone found in other subjects, PJ_9 and TrK_9, indicated no corpus luteum formation, if one may only look at the value of progesterone. However, the levels of estradiol of higher than 150 pg/ml until days 22 of the cycle suggested that there was possibly the presence of presistent follicles. Subject. DN_9 had a rather long length cycle and no rise of progesterone and estradiol levels, suggested that there might be an undevelopment of follicles or the absence of corpus luteum formation.

The low levels of progesterone after Norgestrel treatment found in this study was, however, different from the absence of inhibition observed in Caucasian women (Foss et al., 1968; Wright et al., 1970; Eckstein, 1972 and Kesseru, 1972). It is too early to conclude, due to the lack of published information, that this discrepancy was due to ethical factor or enzymic differences for the progesterone biosynthesis.

One of the most comprehensive accounts from various studies of progesterone levels during pregnancy (Zander, 1955; Sommerville & Desphande, 1958; Short, 1961; Greig et al., 1962; Van der Molen, 1963; Fuchs et al., 1963; Vannone et al., 1968 and 1969 and Johansson, 1969 b) was that of Johansson (1969 b),

who suggested that the placenta does not replace the corpus luteum as the major source of progesterone until at about the 9th week of gestation. From the 9th to the 32nd week, there was a gradual increase in blood progesterone concentration but thereafter no further increment was observed.

Abraham (1971) reported that the serum progesterone levels during pregnancy were 48.40±18.00 ng/ml (range 30-81) in 16-18 weeks of gestation; 98.00±28.00 ng/ml (range 65-174) in 28-30 weeks of gestation and 178.50±48 ng/ml (range 121-290) in 38-40 weeks of gestation.

It was found in the present study that the progesterone level at 4 weeks was 15.70 ng/ml. This value gradually rose to a considerably high level ranging from 173-182 ng/ml (taple 17, page 58) at 38-40 weeks of gestation.

Following the delivery, a rapid fall in serum progesterone levels occurred and within 5 days of this event (table 18, page 60) the levels were in the same range as those found during the luteal phase of menstrual cycle. The mean levels of serum progesterone were 1.60 ng/ml serum after 116 hours of delivery.

The levels of serum progesterone during the normal cycle and pregnancy in this study were well within the range of the values recently described by other investigators, Abraham et al., 1971; Mikhail & de Villa, 1972 and Saxena et al., 1974, using similar radioimmunoassay techniques suggested that the method could possibly be applied for further studies in various fields.